



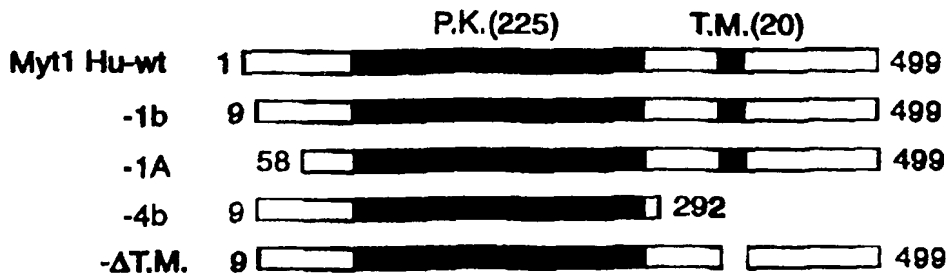
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/54, 15/86, 9/12, 5/10, C12Q 1/48, C07K 16/40, A61K 38/45</b>		<b>A1</b>	(11) International Publication Number: <b>WO 98/21342</b>
			(43) International Publication Date: 22 May 1998 (22.05.98)
(21) International Application Number: PCT/US97/18577 (22) International Filing Date: 15 October 1997 (15.10.97) (30) Priority Data: 60/031,266 15 November 1996 (15.11.96) US (71) Applicant: ONYX PHARMACEUTICALS, INC. [US/US]; 3031 Research Drive, Richmond, CA 94806 (US). (72) Inventors: BOOHER, Robert, N.; 1720 Funston Avenue, San Francisco, CA 94122 (US). FATTAEY, Ali; Apartment 202, 1770 Green Street, San Francisco, CA 94123 (US). (74) Agent: GIOTTA, Gregory; Onyx Pharmaceuticals, Inc., 3031 Research Drive, Richmond, CA 94806 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	

(54) Title: NUCLEIC ACID SEQUENCES THAT ENCODE A CELL GROWTH REGULATORY PROTEIN AND USES THEREOF

## Human Myt1 Transmembrane Region

## pCMV-HA:MYT1 Constructs



## (57) Abstract

Compositions of matter consisting of a family of related nucleic acid sequences that encode proteins, termed Cell Growth Regulatory Proteins, that phosphorylate cell cycle targets, and methods of using the nucleic acid sequences and the proteins encoded thereby, to diagnose and/or treat disease where the Cell Growth Regulatory Proteins have been shown to be involved in the regulation of cell growth. The proteins have a molecular weight of about 54.6 kdaltons.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LI	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NI	Nicaragua	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Cote d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

Nucleic Acid Sequences that Encode a Cell Growth  
Regulatory Protein and Uses Thereof

5

Field of the Invention

The invention described herein relates generally to the field of human disease, and more specifically to treating and diagnosing disease based on nucleic acid sequences that encode a human Cell Growth Regulatory Protein(s) that regulates cell growth by Phosphorylation of cell cycle proteins.

Background

Oncogenic transformation of cells leads to a number of changes in cellular metabolism, physiology, and morphology. One characteristic alteration of oncogenically transformed cells is a loss of responsiveness to constraints on cell proliferation and differentiation normally imposed by the appropriate expression of cell growth regulatory genes. While different types of genetic alterations may all lead to altered expression or function of cell-growth regulatory genes and to abnormal growth, it is generally believed that more than one event is required to lead to neoplastic transformation of a normal cell to a malignant one (Land et al. (1983) Nature 304: 596; Weinberg RA (1989) Cancer Res. 49: 3713). The precise molecular pathways and secondary changes leading to malignant transformation for most cell types are not clear. A number of cases have been reported in which altered expression or activity of some proteins with putative cell-cycle control functions and/or implicated in the formation of functional transcriptional complexes, such as *p53* and *RB*, can lead to loss of proliferation control in cells (Ullrich et al. (1992) J. Biol. Chem. 267: 15259; Holstein et al. (1991) Science 253: 49; Sager R (1992) Curr. Opin. Cell. Biol. 4: 155; Levine et al. (1991) Nature 351: 453).

Regarding cell cycle aspects of oncogenic transformation, several proteins involved in regulating the cell cycle have recently been identified and shown to be critical regulators of mitosis. The progression of a eukaryotic cell through the cell cycle to mitosis involves a number of what appear to be tightly regulated mutually

antagonistic phosphatases and kinases. A key kinase in this process is *cdc2*, which is thought to be responsible for initiating mitosis.

*Cdc2* is involved in a number of downstream mitotic events including metaphase alignment of chromosomes, segregation of sister chromatids in anaphase, and cleavage furrow formation. A large number of proteins involved in these processes are phosphorylated by *cdc2* including histones, lamins and microtubule-associated proteins. See, Nigg, Semin. Cell Biol. 2: 261-270 (1991). Particularly noteworthy is the phosphorylation of caldesmon, an actin-associated protein, which is thought to be necessary for the dissolution of M-phase specific actin cables. Well known changes in nuclear structure also involve *cdc2*. For instance chromatin condensation involves *cdc2* phosphorylation of the histone H1. See, Langan et al. Molec. Cell. Biol. vol. 9: 3860-3868. The dissolution of the nuclear envelope is associated with *cdc2* phosphorylation of lamin B. See, Peter, et al. Cell, vol. 61: 591-602 (1990). Further, during mitosis the nucleolus disappears and this event is also associated with *cdc2* activity.

Considering the importance of *cdc2* to critical cell cycle functions, and therefore to cell growth, one would expect that this enzyme is subject to several significant control mechanisms. Indeed two such mechanisms have been identified and include the phosphorylation of *cdc2* at three sites; tyrosine 15, threonine 14 and threonine 161. Phosphorylation of threonine 161 and dephosphorylation of tyrosine 15 and threonine 14 are necessary for kinase activity. The phosphorylation of tyrosine 15 and threonine 14 is thus a negatively regulator of *cdc2* kinase activity. See, Mueller, P. R., et al. Science, vol. 270, pages 86-90 (1995). An enzyme termed cyclin-dependent kinase (CDK)-activating kinase (CAK) phosphorylates *cdc2* on threonine 161. An enzyme termed Wee1 kinase has been shown to phosphorylate *cdc2* at tyrosine 15 but not threonine 14. See, Mueller, P. R., et al. Mol. Biol. Cell vol. 6 page 119 (1995). Recently, a second enzyme has been identified from *Xenopus* that phosphorylates both amino acids, and has been termed Myt1 for its membrane association, and its capacity to phosphorylate both tyrosine 15 and threonine 14 on *cdc2*. There is significant nucleotide homology between the Wee1 and *Xenopus* Myt1 enzymes.

It is worth noting certain other proteins involved in *cdc2* action. In order for *cdc2* to exhibit kinase activity, and thus phosphorylate its mitotic substrates the phosphates at positions tyrosine 15 and threonine 14 must be removed. This is realized

by the phosphatase cdc25. Cdc25 is, in turn, activated by cdc2 phosphorylation. See, Hoffmann, et al., EMBO J. vol: 12; page 53 (1993). Cdc2 is part of a complex consisting of a cyclin, cyclin B. The phosphorylation events that occur on cdc2 occur after its association with cyclin B

5 The identification and isolation of a human enzyme with kinase activity similar to *Xenopus* Myt1 will have significant medical applications both as an inhibitor of uncontrolled cell growth as displayed by cancer cells, and as a means to identify small molecules with similar activity in drug screening assays. Moreover, the enzyme will have diagnostic applications.

#### 10 Summary of the Invention

A first object of the invention is to describe a family of related isolated nucleic acid sequences that encode proteins with kinase activity, hereinafter referred to as Cell Growth Regulatory Proteins.

15 A second object of the invention is to describe a family of related isolated nucleic acid sequences that encode such Cell Growth Regulatory Proteins having molecular weights ranging from about 45-60 kdaltons.

20 A third object of the invention is to describe an isolated human Cell Growth Regulatory Protein having a calculated molecular weight of about 54.6 kdaltons that phosphorylates Cdc2 of the Cdc2/CyclinB complex at amino acid residues threonine 14 and tyrosine 15, which phosphorylation substantially inhibits the kinase activity of the Cdc2/CyclinB complex.

A fourth object of the invention is to describe isolated nucleic acid sequences that encode protein fragments, or the fragments themselves of Cell Growth Regulatory Protein(s), respectively.

25 A fifth object of the invention is to describe host cells transformed with isolated nucleic acid sequences that encodes Cell Growth Regulatory Protein(s) or fragments thereof.

A sixth object of the invention is to describe vectors that contain isolated nucleic sequences that encode Cell Growth Regulatory Protein(s) or fragments thereof.

30 A seventh object of the invention is to describe complexes consisting of full length or fragments of Cell Growth Regulatory Proteins and cell cycle target proteins.

An eighth object of the invention is to describe methods of diagnosing disease, preferably those involving unwanted cell growth, including cancer, using isolated nucleic acid sequences, or fragments thereof, that encode a Cell Growth Regulatory Protein, or fragments thereof.

5 A ninth object of the invention is a description of antibody that binds to Growth Regulatory Protein, or fragments thereof.

A tenth object of the invention is to describe an assay using isolated nucleic acid sequences that encode a Cell Growth Regulatory Protein, or fragments thereof for identifying compounds that would have therapeutic applications for the treatment of  
10 diseases involving unwanted cell growth, including cancer.

These and other objects of the present invention will become apparent to one of ordinary skill in the art upon reading the description of the various aspects of the invention in the following specification. The foregoing and other aspects of the present invention are explained in greater detail in the drawings, detailed description, and  
15 examples set forth below.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

**Figure 1** shows the cDNA (Seq I. D. No. 1) and amino acid sequence (Seq I. D. No. 1) of the Cell Growth Regulatory Protein described in Example 1.

**Figure 2** shows a diagram of the amino acid sequence (Seq I. D. No. 2) of the  
20 Cell Growth Regulatory Protein described in Example 1 and four truncated Cell Growth Regulatory Protein mutants including deletions of the N-terminus, C-terminus, and the transmembrane segments. The expressed Cell Growth Regulatory Protein proteins contain an N-terminal HA epitope tag and include Cell Growth Regulatory Protein-1b (residues 9-499), Cell Growth Regulatory Protein-1A (residues 58-499), Cell Growth  
25 Regulatory Protein-4b (residues 9-292), and Cell Growth Regulatory Protein-ΔT.M. (deleted transmembrane) (residues 9-499 with residues 379-398 deleted).

### **Detailed Description of the Invention**

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or  
30 patent application was specifically and individually indicated to be incorporated by reference.

### Definitions

At the outset it is worth noting that unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures described below are those well known and commonly employed in the art. Standard techniques are used for recombinant nucleic acid methods, polynucleotide synthesis, and microbial culture and transformation (e.g., electroporation, lipofection). Generally enzymatic reactions and purification steps are performed according to the manufacturer's specifications. The techniques and procedures are generally performed according to conventional methods in the art and various general references (see generally, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd. edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which is incorporated herein by reference) which are provided throughout this document. The nomenclature used herein and the laboratory procedures in analytical chemistry, organic synthetic chemistry, and pharmaceutical formulation described below are those well known and commonly employed in the art. Standard techniques are used for chemical syntheses, chemical analyses, pharmaceutical formulation and delivery, and treatment of patients.

The definitions of the cell cycle, and proteins involved therewith are well known to the skilled practitioner of this art. Briefly, cdc2 and cdc25 are defined as shown by Mueller, P. R., et al. Science, vol. 270, pages 86-90 (1995). Cdc2 associates with cyclin B to form a complex that phosphorylates a number of proteins provided that cdc2 is itself phosphorylated at threonine 161, and dephosphorylated at threonine 14 and tyrosine 15.

In the formulas representing selected specific embodiments of or Cell Growth Regulatory Proteins of the present invention, the amino- and carboxy-terminal groups, although often not specifically shown, will be understood to be in the form they would assume at physiological pH values, unless otherwise specified. Thus, the N-terminal  $H_3^+$  and C-terminal- $O^-$  at physiological pH are understood to be present though not necessarily specified and shown, either in specific examples or in generic formulas. In the polypeptide notation used herein, the left-hand end of the molecule is the amino terminal end and the right-hand end is the carboxy-terminal end, in accordance with

standard usage and convention. Of course, the basic and acid addition salts including those which are formed at nonphysiological Ph values are also included in the compounds of the invention. The amino acid residues described herein are preferably in the "L" isomeric form. Stereoisomers (e.g., D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as  $\alpha,\alpha$ -distributed amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for polypeptides of the present invention, as long as the desired functional property is retained by the polypeptide. For the peptides shown, each encoded residue where appropriate is represented by a three letter designation, corresponding to the trivial name of the conventional amino acid, in keeping with standard polypeptide nomenclature (described in J. Biol. Chem., 243:3552-59 (1969) and adopted at 37 CFR §1.822(b)(2)).

Free functional groups, including those at the carboxy- or amino-terminus, referred to as noninterfering substituents, can also be modified by amidation, acylation or other substitution, which can, for example, change the solubility of the compounds without affecting their activity.

As employed throughout the disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

The term "isolated protein" referred to herein means a protein of cDNA, recombinant RNA, or synthetic origin or some combination thereof, which by virtue of its origin the "isolated protein" (1) is not substantially associated with proteins found in nature, (2) is substantially free of other proteins from the same source, e.g. free of human proteins, (3) may be expressed by a cell from a different species, or (4) does not occur in nature.

The term "naturally-occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

The term "polynucleotide" as referred to herein means a polymeric form of nucleotides of at least 10 bases in length, either ribonucleotides or deoxynucleotides or a



modified form of either type of nucleotide. The term includes single and double stranded forms of DNA.

The term "oligonucleotide" referred to herein includes naturally occurring, and modified nucleotides linked together by naturally occurring, and non-naturally

5 occurring oligonucleotide linkages. Oligonucleotides are a polynucleotide subset with 200 bases or fewer in length. Preferably oligonucleotides are 10 to 60 bases in length and most preferably 12, 13, 14, 15, 16, 17, 18, 19, or 20 to 40 bases in length.

Oligonucleotides are usually single stranded, e.g. for probes; although oligonucleotides may be double stranded, e.g. for use in the construction of a gene mutant.

10 Oligonucleotides of the invention can be either sense or antisense oligonucleotides. The term "naturally occurring nucleotides" referred to herein includes deoxyribonucleotides and ribonucleotides. The term "modified nucleotides" referred to herein includes nucleotides with modified or substituted sugar groups and the like. The term

15 "oligonucleotide linkages" referred to herein includes oligonucleotides linkages such as phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoraniladate, phosphoroamidate, and the like. An oligonucleotide can include a label for detection, if desired.

The term "sequence homology" referred to herein describes the proportion of base matches between two nucleic acid sequences or the proportion amino acid matches  
20 between two amino acid sequences. When sequence homology is expressed as a percentage, e.g., 50%, the percentage denotes the proportion of matches over the length of sequence from Cell Growth Regulatory Protein that is compared to some other sequence. Gaps (in either of the two sequences) are permitted to maximize matching; gap lengths of 15 bases or less are usually used, 6 bases or less are preferred with 2  
25 bases or less more preferred. When using oligonucleotides as probes or treatments the sequence homology between the target nucleic acid and the oligonucleotide sequence is generally not less than 17 target base matches out of 20 possible oligonucleotide base pair matches (85%); preferably not less than 9 matches out of 10 possible base pair matches (90%), and most preferably not less than 19 matches out of 20 possible base pair  
30 matches (95%).

Two amino acid sequences are homologous if there is a partial or complete identity between their sequences. For example, 85% homology means that 85% of the

amino acids are identical when the two sequences are aligned for maximum matching. Gaps (in either of the two sequences being matched) are allowed in maximizing matching; gap lengths of 5 or less are preferred with 2 or less being more preferred. Alternatively and preferably, two protein sequences (or polypeptide sequences derived from them of at least 30 amino acids in length) are homologous, as this term is used herein, if they have an alignment score of at more than 5 (in standard deviation units) using the program ALIGN with the mutation data matrix and a gap penalty of 6 or greater. See Dayhoff, M.O., in Atlas of Protein Sequence and Structure, 1972, volume 5, National Biomedical Research Foundation, pp. 101-110, and Supplement 2 to this volume, pp. 1-10. The two sequences or parts thereof are more preferably homologous if their amino acids are greater than or equal to 50% identical when optimally aligned using the ALIGN program.

As used herein, "substantially pure" means an object species is the predominant species present (i.e., on a molar basis it is more abundant than any other individual species in the composition), and preferably a substantially purified fraction is a composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition will comprise more than about 80 percent of all macromolecular species present in the composition, more preferably more than about 85%, 90%, 95%, and 99%. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species.

The phrases "Cell Growth Regulatory Protein," "Cell Growth Regulatory Peptide," or "Cell Growth Regulatory Polypeptide" refer to proteins or peptides with kinase activity that regulate cell growth by phosphorylation of proteins that control the cell cycle. Each of these definitions is meant to encompass one or more such entities.

The phrases "cell cycle target," or "cell cycle target gene product" refer to a protein which is phosphorylated by a Cell Growth Regulatory Protein and which is involved in controlling cell growth by affecting the cell cycle.. Such "cell cycle targets" may be dimeric; an example is cdc2. Cdc2 requires association with cyclin B to be phosphorylated at threonine 14 and tyrosine 15 by the Cell Growth Regulatory Protein

described herein. Each of these definitions is also meant to encompass one or more such entities.

Chemistry terms herein are used according to conventional usage in the art, as exemplified by The McGraw-Hill Dictionary of Chemical Terms (ed. Parker, S., 1985), McGraw-Hill, San Francisco, incorporated herein by reference.

The production of proteins from cloned genes by genetic engineering is well known. See, e.g. U.S. Patent Number 4,761,371 to Bell *et al.* at column 6, line 3 to column 9, line 65. (The disclosure of all patent references cited herein is to be incorporated herein by reference.) The discussion which follows is accordingly intended as an overview of this field, and is not intended to reflect the full state of the art.

DNA regions are operably linked when they are functionally related to each other. For example: a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked means contiguous and, in the case of leader sequences, contiguous and in reading frame.

Suitable host cells include prokaryotes, yeast cells, or higher eukaryotic cells. Prokaryotes include gram negative or gram positive organisms, for example *Escherichia coli* (*E. coli*) or Bacilli. Higher eukaryotic cells include established cell lines of mammalian origin as described below. Exemplary host cells are DH5a, *E. coli* W3110 (ATCC 27,325), *E. coli* B, *E. coli* X1776 (ATCC 31,537) and *E. coli* 294 (ATCC 31,446). *Pseudomonas* species, *Bacillus* species, and *Serratia marcescens* are also suitable.

In an insect system, *Autographa californica* nuclear polyhydrosis virus (AcNPV) may be used as a vector to express foreign genes. (E.g., see Smith et al., 1983, J. Virol. 46: 584; Smith, U.S. Patent No. 4,215,051). In a specific embodiment described below, Sf9 insect cells are infected with a baculovirus vectors expressing a Cell Growth Regulatory construct with either a 6x histidine tag, myc, or an EE-tag (i.e. Glu-Glu-tag). "E" refers to the amino acid glutamine.

A broad variety of suitable microbial vectors are available. Generally, a microbial vector will contain an origin of replication recognized by the intended host, a promoter which will function in the host and a phenotypic selection gene such as a gene

encoding proteins conferring antibiotic resistance or supplying an autotrophic requirement. Similar constructs will be manufactured for other hosts. *E. coli* is typically transformed using pBR322. See Bolivar *et al.*, *Gene* 2, 95 (1977). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. Expression vectors should contain a promoter which is recognized by the host organism. This generally means a promoter obtained from the intended host. Promoters most commonly used in recombinant microbial expression vectors include the beta-lactamase (penicillinase) and lactose promoter systems (Chang *et al.*, *Nature* 275, 615 (1978); and Goeddel *et al.*, *Nucleic Acids Res.* 8, 4057 (1980) and EPO Application Publication Number 36,776) and the *tac* promoter (H. De Boer *et al.*, *Proc. Natl. Acad. Sci. USA* 80, 21 (1983)). While these are commonly used, other microbial promoters are suitable. Details concerning nucleotide sequences of many promoters have been published, enabling a skilled worker to operably ligate them to DNA encoding Cell Growth Regulatory Protein in plasmid or viral vectors (Siebenlist *et al.*, *Cell* 20, 269, 1980)). The promoter and Shine-Dalgarno (SD) sequence (for prokaryotic host expression) are operably linked to the DNA encoding myt 1, i.e. they are positioned so as to promote transcription of the myt 1 messenger RNA from the DNA. The SD sequence is thought to promote binding of mRNA to the ribosome by the pairing of bases between the SD sequence and the 3' end of *E. coli* 16S rRNA (Steitz *et al.* (1979). In Biological Regulation and Development: Gene Expression (ed. R.F. Goldberg)). To express eukaryotic genes and prokaryotic genes with a weak ribosome-binding site see Sambrook *et al.* (1989) "Expression of cloned genes in *Escherichia coli*." In Molecular Cloning: A Laboratory Manual. Furthermore, a bacterial promoter can include naturally occurring promoters of non-bacterial origin that have the ability to bind bacterial RNA polymerase and initiate transcription. A naturally occurring promoter of non-bacterial origin can also be coupled with a compatible RNA polymerase to produce high levels of expression of some genes in prokaryotes. The bacteriophage T7 RNA polymerase/promoter system is an example of a coupled promoter system (Studier *et al.* (1986) *J. Mol. Biol.* 189:113; Tabor *et al.* (1985) *Proc. Natl. Acad. Sci.* 82:1074). In addition, a hybrid promoter can also be composed of a bacteriophage promoter and an *E. coli* operator region (EPO Pub. No. 267,851).

Cell Growth Regulatory Proteins can be expressed intracellularly. A promoter sequence can be directly linked with a Cell Growth Regulatory gene or a fragment thereof, in which case the first amino acid at the N-terminus will always be a methionine, which is encoded by the ATG start codon. If desired, methionine at the N-terminus can be cleaved from the protein by *in vitro* incubation with cyanogen bromide or by either *in vivo* or *in vitro* incubation with a bacterial methionine N-terminal peptidase (EPO Pub. No. 219,237).

Cell Growth Regulatory Proteins of the instant invention are membrane associated and display a membrane segment. Such proteins can be generated with or without the membrane segment. To facilitate their purification in those instances where the transmembrane segment is not desired the segment can be removed to yield a soluble cytosolic Cell Growth Regulatory Protein using techniques well known in the art.

Eukaryotic microbes such as yeast cultures may be transformed with suitable vectors containing a Cell Growth Regulatory Protein. See, e.g. U.S. Patent Number 4,745,057. *Saccharomyces cerevisiae* is the most commonly used among lower eukaryotic host microorganisms, although a number of other strains are commonly available. Yeast vectors may contain an origin of replication from the 2 micron yeast plasmid or an autonomously replicating sequence (ARS), a promoter, DNA encoding Cell Growth Regulatory Protein, sequences for polyadenylation and transcription termination, and a selection gene.

Suitable promoting sequences in yeast vectors include the promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman *et al.*, *J. Biol. Chem.* 255, 2073 (1980) or other glycolytic enzymes (Hess *et al.*, *J. Adv. Enzyme Reg.* 7, 149 (1968); and Holland *et al.*, *Biochemistry* 17, 4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzman *et al.*, EPO Publication Number 73,657.

Cultures of cells derived from multicellular organisms are a desirable host for recombinant Cell Growth Regulatory Protein synthesis. In principal, any higher

eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. However, mammalian cells are preferred, as illustrated in the Examples. Propagation of such cells in cell culture has become a routine procedure. See Tissue Culture, Academic Press, Kruse and Paterson, editors (1973).

5           The transcriptional and translational control sequences in expression vectors to be used in transforming vertebrate cells are often provided by viral sources. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and Simian Virus 40 (SV40). See, e.g., U.S. Patent Number 4,599,308.

10           An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral source (e.g. Polyoma, Adenovirus, VSV, or BPV), or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter may be sufficient.

#### **Identification of Cell Growth Regulatory Proteins**

15           Cell Growth Regulatory Proteins can be identified using several different techniques for detecting protein-protein interactions. Among the traditional methods which may be employed are co-immunoprecipitation, crosslinking and co-purification through gradients or chromatographic columns of cell lysates or proteins obtained from cell lysates, and to identify proteins in the lysate that phosphorylate target proteins  
20           known to regulate cell growth through the cell cycle (i.e. cell cycle target proteins such as cdc2). Such assays may employ full length cell cycle targets or a peptide. Once isolated, such an intracellular Cell Growth Regulatory Protein can be identified and can, in turn, be used, in conjunction with standard techniques, to identify other proteins with which it interacts. For example, at least a portion of an amino acid sequence of an  
25           intracellular Cell Growth Regulatory Protein which interacts with a cell cycle target protein can be ascertained using techniques well known to those of skill in the art, such as the Edman degradation technique. (See, e.g., Creighton, 1983, "Proteins: Structures and Molecular Principles", W.H. Freeman & Co., N.Y., pp.34-49). The amino acid sequence obtained may be used as a guide for the generation of oligonucleotide  
30           mixtures that can be used to screen for gene sequences encoding such intracellular proteins. Screening may be accomplished, for example, by standard hybridization or PCR techniques. Techniques for the generation of oligonucleotide mixtures and the

screening are well-known. (See, e.g., Ausubel, supra, and PR Protocols: A Guide to Methods and Applications, 1990, Innis, M. et al., eds. Academic Press, Inc., New York).

Additionally, methods may be employed which result in the simultaneous identification of genes which encode the intracellular proteins interacting with . These methods include, for example, probing expression, libraries, in a manner similar to the well known technique of antibody probing of  $\lambda$ gt11 libraries, using labeled protein, or fusion protein, e.g., fused to a marker (e.g., and enzyme, fluor, luminescent protein, or dye), or an Ig-Fc domain.

One method which detects protein interactions *in vivo*, and which does not rely on the kinase activity of Cell Growth Regulatory Protein, is the two-hybrid system, and is described in detail for illustration only and not by way of limitation. This system has been described ( U. S. Patent No. 5, 283, 173 Chien et al., 1991, Proc. Natl. Acad. Sci. USA, 88:9578-9582) and is commercially available from Clontech (Palo Alto, CA). Briefly, utilizing such a system, plasmids are constructed that encode two hybrid proteins: one plasmid consists of nucleotides encoding the DNA-binding domain of a transcription activator protein fused to a Cell Growth Regulatory nucleotide sequence encoding a Cell Growth Regulatory , or peptide or fusion protein, and the other plasmid consists of nucleotides encoding the transcription activator protein's activation domain fused to a cDNA encoding an unknown protein which has been recombined into this plasmid as a part of the cDNA library. The DNA-binding domain fusion plasmid and the cDNA library are transformed into a strain of the yeast Saccharomyces cerevisiae that contains a reporter gene (e.g., HBS or lacZ) whose regulatory region contain the transcription activator's binding site. Either hybrid protein alone cannot activate transcription of the reporter gene; the DNA-binding domain hybrid cannot because it does not provide activation function, and the activation domain hybrid cannot because it cannot localize to the activator's binding sites. Interaction of the two hybrid proteins reconstitutes the functional activator protein and results in expression of the reporter gene, which is detected by an assay for the reporter gene product.

The two-hybrid system or related methodology may be used to screen activation domain libraries for proteins that interact with the "bait" gene product. By way of example, and not by way of limitation, cell cycle proteins that regulate cell growth ((i.e. cell cycle target proteins such as cdc2), or a peptide, or fusion protein derived

therefrom may be used as the bait gene product. Total genomic or cDNA sequences are fused to the DNA encoding an activation domain. This library and a plasmid encoding a hybrid of a bait cell cycle target gene product fused to the DNA-binding domain are cotransformed into a yeast reporter strain, and the resulting transformants are screened  
5 for those that express the reporter gene. For example, and not by way of limitation, a bait gene sequence, such as the open reading frame of a cell cycle target protein, or a domain of cell cycle target protein (i.e. a peptide that contains among other amino acids, threonine 14 and tyrosine 15 of cdc2) can be cloned into a vector such that it is translationally fused to the DNA encoding the DNA-binding domain of the GAL4  
10 protein. These colonies are purified and the library plasmids responsible for reporter gene expression are isolated. DNA sequencing is then used to identify the proteins encoded by the library plasmids.

A cDNA library of the cell line from which proteins that interact with bait cell cycle target gene product are to be detected can be made using methods routinely  
15 practiced in the art. According to the particular system described herein, for example, the cDNA fragments can be inserted into a vector such that they are translationally fused to the transcriptional activation domain of GAL4. This library can be co-transfected along with the bait cell cycle target gene-GAL4 fusion plasmid into a yeast strain which contains a lacZ gene driven by a promoter which contains GAL4 activation  
20 sequence. A cDNA encoded protein, fused to GAL4 transcriptional activation domain, that interacts with bait cycle target gene product will reconstitute an active GAL4 protein and thereby drive expression of the HIS3 gene. Colonies which express HIS3 can be detected by their growth on petri dishes containing semi-solid agar based media lacking histidine. The cDNA can then be purified from these strains, and used to  
25 produce and isolate the bait cycle target gene-interacting protein using techniques routinely practiced in the art.

Whenever a protein is isolated using the two-hybrid assay, an independent assay to ascertain whether the protein has kinase activity is performed. Such assays are well known in the art, and an example is described by Mueller, P. R. et al., Mol. Biol. Cell,  
30 vol. 6, page 119 (1995).



### Cell Growth Regulatory Protein cDNA

The cDNA, and deduced amino acid sequence, of a representative Cell Growth Regulatory Protein is shown in Figure 1. The cDNA encodes a protein that has a calculated molecular weight of 54.6 kd.

5 The Cell Growth Regulatory Protein nucleotide sequences of the invention include: (a) the human cDNA sequence shown in Figure 1 or contained in the cDNA clone as deposited with the American Type Culture Collection under accession number 98169; (b) and any non-Xenopus nucleotide sequence that hybridizes to the complement of the DNA sequence shown in Figure 1 or contained in the cDNA clone as deposited  
10 with the ATCC accession number 98169 under highly stringent conditions, e.g., hybridization to filter-bound DNA in 0.5 M NaHPO<sub>4</sub>, 7% sodium dodecyl sulfate (SDS), 1mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3) and encodes a functionally  
15 equivalent gene product; and (c) any non-Xenopus nucleotide sequence that hybridizes to the complement of the DNA sequences that encode the amino acid sequence shown in Figure 1 or contained in the cDNA clone as deposited with the ATCC 98169 under less stringent conditions, such as moderately stringent conditions, e.g., washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel et al., 1989, supra), yet which still encodes a  
20 functionally equivalent Cell Growth Regulatory Protein gene product. Functional equivalents include naturally occurring Cell Growth Regulatory Protein genes present in other species, excluding Xenopus, and mutant Cell Growth Regulatory Protein genes whether naturally occurring or engineered which retain at least some of the functional activities of a Cell Growth Regulatory Protein (i.e., binding to ). The invention also  
25 includes degenerate variants of sequences (a) through (c).

The invention also includes nucleic acid molecules, preferably DNA molecules, that hybridize to, and are therefore the complements of, the nucleotide sequences (a) through (c), in the preceding paragraph. Such hybridization conditions may be highly stringent or less highly stringent, as described above. In instances wherein the nucleic  
30 acid molecules are deoxyoligonucleotides ("oligos"), highly stringent conditions may refer, e.g., to washing in 6xSSC/0.05% sodium pyrophosphate at 37°C (for 14-base oligos), 48°C (for 17-base oligos), 55°C (for 20-base oligos), and 60°C (for 23-base

oligos). These nucleic acid molecules may encode or act as Cell Growth Regulatory gene antisense molecules, useful, for example, in gene regulation (for and/or as antisense primers in amplification reactions of Cell Growth Regulatory gene nucleic acid sequences). Such sequences may be used as part of ribozyme and/or triple helix sequences, also useful for Cell Growth Regulatory gene regulation. Still further, such molecules may be used as components of diagnostic methods whereby, for example, the presence of a particular Cell Growth Regulatory Protein allele associated with uncontrolled cell growth (i.e. cancer) may be detected.

Further, it will be appreciated by one skilled in the art that a Cell Growth Regulatory Protein gene homolog may be isolated from nucleic acid of an organism of interest by performing PCR using two degenerate oligonucleotide primer pools designed on the basis of amino acid sequences within the Cell Growth Regulatory gene product disclosed herein. The template for the reaction may be cDNA obtained by reverse transcription of mRNA prepared from, for example, human or non-human cell lines or cell types, such as breast or ovarian cells, known or suspected to express a Cell Growth Regulatory gene allele.

The PCR product may be subcloned and sequenced to ensure that the amplified sequences represent the sequences of a Cell Growth Regulatory gene. The PCR fragment may then be used to isolate a full length cDNA clone by a variety of methods. For example, the amplified fragment may be labeled and used to screen a cDNA library, such as a bacteriophage cDNA library. Alternatively, the labeled fragment may be used to isolate genomic clones via the screening of a genomic library.

PCR technology may also be utilized to isolate full length cDNA sequences. For example, RNA may be isolated, following standard procedures, from an appropriate cellular source (i.e., one known, or suspected, to express a Cell Growth Regulatory Protein gene, such as, for example, from breast or ovarian cells). A reverse transcription reaction may be performed on the RNA using an oligonucleotide primer specific for the most 5' end of the amplified fragment for the priming of first strand synthesis. The resulting RNA/DNA hybrid may then be "tailed" with guanines using a standard terminal transferase reaction, the hybrid may be digested with RNAase H, and second strand synthesis may then be primed with a poly-C primer. Thus, cDNA sequences

upstream of the amplified fragment may easily be isolated. For a review of cloning strategies which may be used, see e.g., Sambrook et al., 1989, supra.

A cDNA of a mutant Cell Growth Regulatory Protein gene may also be isolated, for example, by using PCR. In this case, the first cDNA strand may be synthesized by hybridizing an oligo-dT oligonucleotide to mRNA isolated from cells known or suspected to be expressed in an individual putatively carrying the mutant Cell Growth Regulatory allele, and by extending the new strand with reverse transcriptase. The second strand of the cDNA is then synthesized using an oligonucleotide that hybridizes specifically to the 5' end of the normal gene. Using these two primers, the product is then amplified via PCR, cloned into a suitable vector, and subjected to DNA sequence analysis through methods well known to those of skill in the art. By comparing the DNA sequence of the mutant Cell Growth Regulatory allele to that of the normal Cell Growth Regulatory allele, the mutation(s) responsible for the loss or alteration of function of the mutant Cell Growth Regulatory gene product can be ascertained.

A genomic library can be constructed using DNA obtained from an individual suspected of or known to carry the mutant Cell Growth Regulatory allele, or a cDNA library can be constructed using RNA from a cell type known, or suspected, to express the mutant Cell Growth Regulatory allele. The normal Cell Growth Regulatory gene or any suitable fragment thereof may then be labeled and used as a probe to identify the corresponding mutant Cell Growth Regulatory allele in such libraries. Clones containing the mutant Cell Growth Regulatory gene sequences may then be purified and subjected to sequence analysis according to methods well known to those of skill in the art.

Additionally, an expression library can be constructed utilizing cDNA synthesized from, for example, RNA isolated from a cell type known, or suspected, to express a mutant Cell Growth Regulatory allele in an individual suspected of or known to carry such a mutant allele. In this manner, gene products made by the putatively mutant cell type may be expressed and screened using standard antibody screening techniques in conjunction with antibodies raised against the normal Cell Growth Regulatory gene product, as described, below. (For screening techniques, see, for example, Harlow, E. and Lane, eds., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Press, Cold Spring Harbor.) Additionally, screening can be

accomplished by screening with labeled fusion proteins. In cases where a Cell Growth Regulatory mutation results in an expressed gene product with altered function (e.g., as a result of a missense or a frameshift mutation), a polyclonal set of antibodies to a Cell Growth Regulatory Protein are likely to cross-react with the Cell Growth Regulatory mutant. Library clones detected via their reaction with such labeled antibodies can be purified and subjected to sequence analysis according to methods well known to those of skill in the art.

The invention also encompasses nucleotide sequences that encode peptide fragments of a Cell Growth Regulatory Protein, truncated Cell Growth Regulatory Proteins, and fusion proteins thereof. Nucleotides encoding fusion proteins may include but are not limited to full length Cell Growth Regulatory Protein, truncated Cell Growth Regulatory Proteins or peptide fragments to an unrelated protein or peptide, such as for example, an epitope tag which aids in purification or detection of the resulting fusion protein; or an enzyme, fluorescent protein, luminescent protein which can be used as a marker. The preferred epitope tag is glu-glu as described by Grussenmyer, T., et al., Proc. Natl. Acad. Sci. U. S. A. vol. 82, pp. 7952-7954 (1985).

The invention also encompasses (a) DNA vectors that contain any of the foregoing Cell Growth Regulatory Protein coding sequences and/or their complements (i.e., antisense); (b) DNA expression vectors that contain any of the foregoing Cell Growth Regulatory Protein coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences; and (c) genetically engineered host cells that contain any of the foregoing Cell Growth Regulatory Protein coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences in the host cell. As used herein, regulatory elements include but are not limited to inducible and non-inducible promoters, enhancers, operators and other elements known to those skilled in the art that drive and regulate expression. Such regulatory elements include but are not limited to the T7 promoter, baculovirus promoter, cytomegalovirus hCMV immediate early gene, the early or late promoters of SV40 adenovirus, the lac system, the trp system, the TAC system, the TRC system, the major operator and promoter regions of phage A, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase, the promoters of acid phosphatase, and the promoters of the yeast-mating factors.

### Cell Growth Regulatory Proteins

As mentioned above, Figure 1 shows the cDNA, and deduced amino acid sequence, of a representative Cell Growth Regulatory Protein. The protein has a calculated molecular weight 54.6.

5 The invention Cell Growth Regulatory Proteins, peptide fragments, mutated, truncated or deleted forms thereof and fusion proteins of these can be prepared for a variety of uses, including but not limited to the generation of antibodies, as reagents in diagnostic assays, the identification and/or the interaction with other cellular gene products involved in cell growth, as reagents in assays for screening for compounds  
10 that can be used in the treatment of unwanted cell growth disorders, including but not limited to cancer, and as pharmaceutical reagents useful in the treatment of such diseases.

The Cell Growth Regulatory Protein amino acid sequences of the invention include the amino acid sequence shown in FIG. 1, or the amino acid sequence encoded  
15 by the cDNA clone, as deposited with the ATCC, accession number 98169. Further, Cell Growth Regulatory Proteins of other species, excluding *Xenopus*, are encompassed by the invention. In fact, any Cell Growth Regulatory Protein encoded by the cDNAs described herein, are within the scope of the invention.

The invention also encompasses proteins that are functionally equivalent to the  
20 Cell Growth Regulatory Protein encoded by the nucleotide sequence described above and shown in Fig. 1, as judged by any of a number of criteria, including but not limited to the ability to bind to and phosphorylate a cell cycle target, and a change in cellular growth properties or change in phenotype when the Cell Growth Regulatory Protein equivalent is present in an appropriate cell type (i.e. an actively growing cell). Such  
25 functionally equivalent Cell Growth Regulatory Protein proteins include but are not limited to additions or substitutions of amino acid residues within the amino acid sequence encoded by the Cell Growth Regulatory Protein nucleotide sequences described above, but which result in a silent change, thus producing a functionally equivalent gene product. Amino acid substitutions may be made on the basis of  
30 similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine,

tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

5           While random mutations can be made to Cell Growth Regulatory Protein DNA (using random mutagenesis techniques well known to those skilled in the art) and the resulting mutant Cell Growth Regulatory Proteins tested for activity, site-directed mutations of the Cell Growth Regulatory Protein coding sequence can be engineered (using site-directed mutagenesis techniques well known to those skilled in the art) to  
10   generate mutant Cell Growth Regulatory Proteins with increased function, e.g., altered binding affinity for .

          For example, mutant Cell Growth Regulatory Proteins can be engineered so that regions of interspecies identity are maintained, whereas the variable residues are altered, e.g., by deletion or insertion of an amino acid residue(s) or by substitution of  
15   one or more different amino acid residues. Conservative alterations at the variable positions can be engineered in order to produce a mutant Cell Growth Regulatory Protein that retains function. Non-conservative changes can be engineered at these variable positions to alter function. Alternatively, where alteration of function is desired, deletion or non-conservative alterations of the conserved regions can be  
20   engineered. One of skill in the art may easily test such mutant or deleted Cell Growth Regulatory Proteins for these alterations in function using the teachings presented herein.

          Other mutations to a Cell Growth Regulatory Protein coding sequence can be made to generate Cell Growth Regulatory Proteins that are better suited for expression,  
25   scale up, etc. in the host cells chosen. For example, the triplet code for each amino acid can be modified to conform more closely to the preferential codon usage of the host cell's translational machinery. Also, the coding sequence can be modified to omit the transmembrane region, thus providing a soluble cytosolic Cell Growth Regulatory Protein.

30           Peptides corresponding to one or more domains (or a portion of a domain) of a Cell Growth Regulatory Protein, truncated or deleted Cell Growth Regulatory Proteins (e.g., Cell Growth Regulatory Proteins in which a portions of one or more of the above

domains are deleted) as well as fusion proteins in which the full length of a Cell Growth Regulatory Protein, a Cell Growth Regulatory Protein peptide or truncated Cell Growth Regulatory Protein is fused to an unrelated protein are also within the scope of the invention and can be designed on the basis of a Cell Growth Regulatory

5 Protein nucleotide and Cell Growth Regulatory Protein amino acid sequences disclosed in this Section and above. Such fusion proteins include but are not limited to fusions to an epitope tag (such as is exemplified herein); or fusions to an enzyme, fluorescent protein, or luminescent protein which provide a marker function.

While the Cell Growth Regulatory Proteins and peptides can be chemically  
10 synthesized (*e.g.*, see Creighton, 1983, *Proteins: Structures and Molecular Principles*, W.H. Freeman & Co., N.Y.), large polypeptides derived from the Cell Growth Regulatory Protein and the full length Cell Growth Regulatory Protein itself may advantageously be produced by recombinant DNA technology using techniques well known in the art for expressing nucleic acid containing Cell Growth Regulatory Protein  
15 gene sequences and/or coding sequences. Such methods can be used to construct expression vectors containing the Cell Growth Regulatory Protein nucleotide sequences described above and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. See, for example, the  
20 techniques described in Sambrook et al., 1989, *supra*, and Ausubel et al., 1989, *supra*. Alternatively, RNA capable of encoding Cell Growth Regulatory Protein nucleotide sequences may be chemically synthesized using, for example, synthesizers. See, for example, the techniques described in "Oligonucleotide Synthesis", 1984, Gait, M.J. ed., IRL Press, Oxford, which is incorporated by reference herein in its entirety.

25 A variety of host-expression vector systems may be utilized to express the Cell Growth Regulatory Protein nucleotide sequences of the invention. Where a Cell Growth Regulatory Protein peptide or polypeptide is a soluble derivative the peptide or polypeptide can be recovered from the culture, *i.e.*, from the host cell in cases where the Cell Growth Regulatory Protein peptide or polypeptide is not secreted, and from  
30 the culture media in cases where a Cell Growth Regulatory Protein peptide or polypeptide is secreted by the cells. However, such engineered host cells themselves may be used in situations where it is important not only to retain the structural and

functional characteristics of a Cell Growth Regulatory Protein, but to assess biological activity, e.g., in drug screening assays.

The expression systems that may be used for purposes of the invention include but are not limited to microorganisms such retrovirus, bacteria (e.g., E. coli, B. subtilis) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing Cell Growth Regulatory Protein nucleotide sequences; yeast (e.g., Saccharomyces, Pichia) transformed with recombinant yeast expression vectors containing the Cell Growth Regulatory Protein nucleotide sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the Cell Growth Regulatory Protein sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing Cell Growth Regulatory Protein nucleotide sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3, U937) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the Cell Growth Regulatory gene product being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of Cell Growth Regulatory Protein or for raising antibodies to the Cell Growth Regulatory Protein, for example, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the E. coli expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which the Cell Growth Regulatory coding sequence may be ligated individually into the vector in frame with the lacZ coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). If the inserted sequence encodes a relatively small polypeptide (less than 25 kD), such fusion proteins are generally soluble and can easily be purified from lysed cells by adsorption



to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety. Alternatively, if the resulting fusion protein is insoluble and forms inclusion bodies in the host cell, the inclusion bodies may be purified and the recombinant protein solubilized using techniques well known to one of skill in the art.

In an insect system, Autographa californica nuclear polyhedrosis virus (AcNPV) may be used as a vector to express foreign genes. (E.g., see Smith et al., 1983, J. Virol. 46: 584; Smith, U.S. Patent No. 4,215,051). In a specific embodiment described below, Sf9 insect cells are infected with a baculovirus vectors expressing either a 6 x HIS-tagged construct, or an (EE)-tagged Cell Growth Regulatory Protein construct.

In mammalian host cells, a number of viral-based expression systems may be utilized. Specific embodiments described more fully below express tagged Cell Growth Regulatory Protein cDNA sequences using a CMV promoter to transiently express recombinant protein in U937 cells or in Cos-7 cells. Alternatively, retroviral vector systems well known in the art may be used to insert the recombinant expression construct into host cells. For example, retroviral vector systems for transducing hematopoietic cells are described in published PCT applications WO 96/09400 and WO 94/29438.

In cases where an adenovirus is used as an expression vector, the Cell Growth Regulatory Protein nucleotide sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the Cell Growth Regulatory gene product in infected hosts. (E.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted Cell Growth Regulatory Protein nucleotide sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire Cell Growth Regulatory Protein gene or cDNA, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be

needed. However, in cases where only a portion of the Cell Growth Regulatory Protein coding sequence is inserted, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (See Bittner et al., 1987, Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and U937 cells.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the Cell Growth Regulatory Protein sequences described above may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the Cell

Growth Regulatory Protein gene. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the Cell Growth Regulatory Protein gene product.

A number of selection systems may be used, including but not limited to the  
5 herpes simplex virus thymidine kinase (Wigler et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy et al., 1980, Cell 22:817) genes can be employed in tk<sup>-</sup>, hgprt<sup>-</sup> or aprt<sup>-</sup> cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which  
10 confers resistance to methotrexate (Wigler et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al., 1981, J. Mol. Biol. 150:1); and hyg<sup>r</sup>, which confers resistance to hygromycin (Santerre et al.,  
15 1984, Gene 30:147).

The Cell Growth Regulatory Protein gene products can also be expressed in transgenic animals. Animals of any species, including, but not limited to mice, rats, rabbits, guinea pigs, pigs, micro-pigs, goats, and non-human primates, e.g., baboons, monkeys, and chimpanzees may be used to generate Cell Growth Regulatory Protein  
20 transgenic animals.

Any technique known in the art may be used to introduce a Cell Growth Regulatory Protein transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to pronuclear microinjection (Hoppe, P.C. and Wagner, T.E., 1989, U.S. Pat. No. 4,873,191); retrovirus mediated gene  
25 transfer into germ lines (Van der Putten et al., 1985, Proc. Natl. Acad. Sci., USA 82:6148-6152); gene targeting in embryonic stem cells (Thompson et al., 1989, Cell 56:313-321); electroporation of embryos (Lo, 1983, Mol Cell Biol. 3:1803-1814); and sperm-mediated gene transfer (Lavitrano et al., 1989, Cell 57:717-723); etc. For a review of such techniques, see Gordon, 1989, Transgenic Animals, Intl. Rev. Cytol. 115:171-229, which  
30 is incorporated by reference herein in its entirety.

The present invention provides for transgenic animals that carry the transgene in some, but not all their cells, i.e., mosaic animals. The transgene may be integrated as

a single transgene or in concatamers, e.g., head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko et al. (Lasko, M. et al., 1992, Proc. Natl. Acad. Sci. USA 89: 6232-6236). The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. When it is desired that the Cell Growth Regulatory Protein transgene be integrated into the chromosomal site of the endogenous Cell Growth Regulatory Protein gene, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous Cell Growth Regulatory Protein gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous Cell Growth Regulatory Protein gene. In this way, the expression of the endogenous Cell Growth Regulatory Protein gene may also be eliminated by inserting non-functional sequences into the endogenous gene. The transgene may also be selectively introduced into a particular cell type, thus inactivating the endogenous Cell Growth Regulatory Protein gene in only that cell type, by following, for example, the teaching of Gu et al. (Gu et al., 1994, Science 265: 103-106). The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. Once transgenic animals have been generated, the expression of the recombinant Cell Growth Regulatory gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to assay whether integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include but are not limited to Northern blot analysis of cell type samples obtained from the animal, *in situ* hybridization analysis, and RT-PCR. Samples of Cell Growth Regulatory Protein gene-expressing tissue, may also be evaluated immunocytochemically using antibodies specific for the Cell Growth Regulatory Protein transgene product, as described below.

### Antibodies to Cell Growth Regulatory Proteins

Antibodies that specifically recognize one or more epitopes of a Cell Growth Regulatory Protein, or epitopes of conserved variants, or peptide fragments are also encompassed by the invention. Such antibodies include but are not limited to  
5 polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')<sub>2</sub> fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above.

The antibodies of the invention may be used, for example, in the detection of the  
10 Cell Growth Regulatory Protein in a biological sample and may, therefore, be utilized as part of a diagnostic or prognostic technique whereby patients may be tested for abnormal amounts of these proteins. Such antibodies may also be utilized in conjunction with, for example, compound screening schemes, as described herein for the evaluation of the effect of test compounds on expression and/or activity of the Cell  
15 Growth Regulatory Protein. Additionally, such antibodies can be used in conjunction with the gene therapy techniques described herein, to, for example, evaluate the normal and/or engineered Cell Growth Regulatory Protein expressing cells prior to their introduction into the patient. Such antibodies may additionally be used as a method for the inhibition of abnormal Cell Growth Regulatory Protein activity.

For the production of antibodies, various host animals may be immunized by  
20 injection with a Cell Growth Regulatory Protein, a Cell Growth Regulatory Protein peptide, truncated Cell Growth Regulatory Protein polypeptides, functional equivalents of the Cell Growth Regulatory Protein or mutants of the Cell Growth Regulatory Protein. Such host animals may include but are not limited to rabbits, mice,  
25 and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human  
30 adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, may be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to the hybridoma technique of Kohler and Milstein, (1975, Nature 256:495-497; and U.S. Patent No. 4,376,110), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today 4:72; Cole et al., 1983, Proc. Natl. Acad. Sci. USA 80:2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies And Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated *in vitro* or *in vivo*. Production of high titers of mAbs *in vivo* makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci. USA, 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a mAb and a human immunoglobulin constant region.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778; Bird, 1988, Science 242:423-426; Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward et al., 1989, Nature 334:544-546) can be adapted to produce single chain antibodies against Cell Growth Regulatory Protein gene products. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')<sub>2</sub> fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')<sub>2</sub> fragments. Alternatively, Fab expression libraries may be constructed (Huse et

al., 1989, Science, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Antibodies to the Cell Growth Regulatory Protein can, in turn, be utilized to generate anti-idiotypic antibodies that "mimic" the Cell Growth Regulatory Protein using techniques well known to those skilled in the art. (See, e.g., Greenspan & Bona, 1993, FASEB J 7(5):437-444; and Nissinoff, 1991, J. Immunol. 147(8):2429-2438).

### Assays for Compounds that Affect Cell

#### Growth Regulatory Protein Activity

As is shown in more detail in the examples, one property of a Cell Growth Regulatory Protein is the capacity to phosphorylate certain proteins that regulate the cell cycle. Indeed, the Cell Growth Regulatory Protein exemplified in Figure 1 phosphorylates cdc2 at tyrosine 15 and threonine 14, which phosphorylation blocks cells from entering mitosis. Thus, compounds that affect the phosphorylation of a cell cycle target protein by a Cell Growth Regulatory Protein(s) will affect the cell cycle, and hence cell growth. The extent of the effect will, in large part, depend on the chemical properties of the compounds tested. Some may strongly disrupt the interaction of the cell cycle target protein with a Cell Growth Regulatory Protein, while others would have a minimal effect. The former would be reflected in altered cell growth, while the latter would not. The converse is also true, certain compounds may strengthen the interaction of with Cell Growth Regulatory Proteins, in which case the opposite biological effect would be anticipated. Thus, it is highly desirable to assay for compounds that affect interactions of cell cycle target proteins with Cell Growth Regulatory Proteins.

The basic principle of the assay systems used to identify such compounds that affects interactions of Cell Growth Regulatory Proteins involves preparing a reaction mixture containing cell cycle target protein (i.e. cdc2 complexed to an appropriate cyclin, preferably cyclin B), polypeptide, peptide or fusion protein of the same, as described above, and a Cell Growth Regulatory Protein under conditions and for a time sufficient to allow the two to interact and for the cell cycle target protein to become phosphorylated. In order to test a compound for activity, either inhibitory or stimulatory, the reaction mixture is prepared in the presence and absence of the test compound. Alternatively, the test compound may be initially included in the reaction

mixture, or may be added at a time subsequent to the addition of the cell cycle target protein and a Cell Growth Regulatory Protein. Control reaction mixtures are incubated without the test compound or with a placebo. The amount or rate of phosphorylation of the cell cycle target protein caused by the Cell Growth Regulatory Protein is then  
5 detected. An increase in phosphorylation over the control reaction mixture indicates that the compound interacts with the reactants to enhance phosphorylation of the cell cycle target protein. Controls can be run to ensure that the test compounds are not causing dephosphorylation of the cell cycle target protein. Such compounds would be useful since they would mimic or enhance the effect of Cell Growth Regulatory  
10 Proteins, and thus have applications as anticancer therapeutics. Additionally, phosphorylation in reaction mixtures containing the test compound and normal, or wild type, Cell Growth Regulatory Protein may also be compared to phosphorylation in reaction mixtures containing the test compound and a mutant Cell Growth Regulatory Protein. This comparison may be important in those cases where it is desirable to  
15 identify compounds that affect phosphorylation of cell cycle target proteins by mutant but not normal Cell Growth Regulatory Protein.

The assay for compounds that interfere with, or stimulate the phosphorylation of a cell cycle target protein by a Cell Growth Regulatory Protein can be conducted in a heterogeneous or homogeneous format. Heterogeneous assays will generally involve  
20 anchoring the cell cycle target protein onto a solid phase and detecting phosphorylated complexes anchored on the solid phase at the end of the reaction. In homogeneous assays, the entire reaction is carried out in a liquid phase. In either approach, the order of addition of reactants can be varied to obtain different information about the compounds being tested. For example, test compounds that interfere with the  
25 interaction by competition can be identified by conducting the reaction in the presence of the test substance; i.e., by adding the test substance to the reaction mixture prior to or simultaneously with the cell cycle target protein and interactive Cell Growth Regulatory Protein. Alternatively, test compounds that disrupt preformed complexes, e.g. compounds with higher binding constants that displace one of the components  
30 from the complex, can be tested by adding the test compound to the reaction mixture after complexes have been formed. Representative formats are described briefly below.



In a heterogeneous assay system, the cell cycle target protein is anchored onto a solid surface, while the non-anchored Cell Cycle Regulatory Protein is used to phosphorylate the target protein. In practice, microtiter plates are conveniently utilized. The anchored species may be immobilized by non-covalent or covalent attachments. Non-covalent attachment may be accomplished simply by coating the solid surface with a solution of the cell cycle target protein and drying. Alternatively, an immobilized antibody specific for the species to be anchored, that is, the cell cycle target protein, may be used to anchor it to the solid surface. The surfaces may be prepared in advance and stored:

In order to conduct the assay, the partner of the immobilized species, that is, the Cell Cycle Regulatory Protein, is exposed under proper reaction conditions to the coated surface with or without the test compound. After the reaction is complete, unreacted components are removed (e.g., by washing) and any phosphorylated complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Generally, radiolabelled phosphate transferred from gamma-labeled P-AIP is measured. Depending upon the order of addition of reaction components, test compounds which inhibit complex formation or which disrupt preformed complexes can be detected.

Alternatively, the reaction can be conducted in a liquid phase in the presence or absence of the test compound, the reaction products separated from unreacted components, and complexes detected; e.g., using an immobilized antibody specific for one of the binding components to anchor any complexes formed in solution, and a labeled antibody specific for the other partner to detect anchored complexes. Again, depending upon the order of addition of reactants to the liquid phase, test compounds which inhibit complex or which disrupt preformed complexes can be identified.

Alternate embodiments of the homogeneous assay are shown in U.S. Patent No. 4,109,496 by Rubenstein.

In a particular embodiment, a fusion protein can be prepared for immobilization. For example, a cell cycle target protein, or a peptide fragment therefrom, can be fused to a glutathione-S-transferase (GST) gene using a fusion vector, such as pGEX-5X-1, in such a manner that its ability to be phosphorylated is maintained in the resulting fusion

protein. In a heterogeneous assay, *e.g.*, the GST- fusion protein can be anchored to glutathione-agarose beads. The Cell Growth Regulatory Protein can then be added in the presence or absence of the test compound in a manner that allows binding and phosphorylation to occur. At the end of the reaction period, unbound material can be washed away, and the labeled cell cycle target protein detected. The interaction between the cell cycle target protein and the Cell Growth Regulatory Protein can be detected by measuring the amount of radioactivity that remains associated with the glutathione-agarose beads. A successful inhibition of the interaction by the test compound will result in a decrease in measured radioactivity.

Alternatively, the GST- fusion cell cycle target protein and the Cell Growth Regulatory Protein can be mixed together in liquid in the absence of the solid glutathione-agarose beads. The test compound can be added either during or after the species are allowed to interact. This mixture can then be added to the glutathione-agarose beads and unbound material is washed away. Again the extent of inhibition of the cell cycle target protein/Cell Growth Regulatory Protein interaction can be detected by measuring the radioactivity associated with the beads.

In another embodiment of the invention, these same techniques can be employed using peptide fragments that correspond to the phosphorylation domains of the cell cycle target protein. Any number of methods routinely practiced in the art can be used to identify and isolate the domains. Such domains are discussed more fully in the examples, below, and for *cdc2*, consist of the ATP binding domain. These methods include, but are not limited to mutagenesis of the gene encoding one of the proteins and screening for disruption of binding in a co-immunoprecipitation assay.

Using the above described approach, Cell Growth Regulatory Protein domains involved in phosphorylation cell cycle target proteins can be identified. Compensating mutations in the gene can then be selected. Sequence analysis of the genes encoding the respective proteins will reveal the mutations that correspond to the region of the protein involved in the phosphorylation reaction. The two hybrid assay may also be used, as discussed more fully in the examples below. For instance, once the gene coding for a Cell Growth Regulatory Protein is obtained, short gene segments can be engineered to express peptide fragments of the protein, which can then be tested for phosphorylation activity and purified or synthesized.

### Effective Dose

Toxicity and therapeutic efficacy of compounds identified above that affect the interaction of cell cycle target proteins with Cell Growth Regulatory Proteins, and thus affect cell growth can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD<sub>50</sub> (the dose lethal to 50% of the population) and the ED<sub>50</sub> (the dose therapeutically effective in 50% of the population). Numerous model systems are known to the skilled practitioner of the art that can be employed to test the cell growth properties of the instant compounds including growth of cells in soft agar, and effect on tumors *in vivo*. Such experiments can be conducted on cells co-transfected with and Cell Growth Regulatory Proteins.

The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD<sub>50</sub>/ED<sub>50</sub>. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED<sub>50</sub> with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC<sub>50</sub> (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

The Examples which follow are illustrative of specific embodiments of the invention, and various uses thereof. They are set forth for explanatory purposes only, and are not to be taken as limiting the invention

### Example 1

#### Cloning of cDNA that Encodes Cell

#### Growth Regulatory Protein

A Cell Growth Regulatory Protein was identified in a human placental cDNA library as follows. A TBLASTN search of the dbEST was performed using the amino acid sequence encoded by Xenopus Cell Growth Regulatory Protein. See, Mueller et al (1995) Science 270, pp 86-90. As expected, the majority of high-scoring matches were protein kinases, owing to the extensive conservation of residues within the catalytic domain of protein kinases (Hanks et al., (1988) Science 42-52). However, two nearly identical human ESTs (dbEST Id: 336264 and 335241) were found to match significantly amino acid tracts within the C-terminal (non-catalytic domain) 130 residues of the Xenopus-Cell Growth Regulatory Protein kinase. A 325-bp DNA fragment corresponding to an internal region of EST clones 335241 and 336264 was PCR-generated, using oligos 5'-AGCAGCCTCTCCAGCAACTGG-3' and 5'-CAGAGAAGACCATGGAGTTCC-3' (5' and 3' primers, respectively) and the first strand cDNA synthesis of fetal brain RNA (Clontech) as template. This DNA fragment was <sup>32</sup>P-labeled by random priming (Pharmacia) and used as a probe to screen approximately 7 × 10<sup>5</sup> clones of a human placental Lambda ZAPII cDNA library (Stratagene Corporation). Of the 9 positives isolated, clone 16 was found to contain the longest cDNA insert, a 1.98-kb cDNA, and the complete DNA sequence of this clone was determined using an ABI sequence. The 1.98-kb cDNA is approximately the size of the Cell Growth Regulatory Protein transcript observed by Northern blotting. Sequence analysis of this clone revealed a 1497-bp open reading frame that is predicted to encode a 499-aa kinase with a calculated molecular mass of 54.6 kDa. We cannot exclude the possibility that this cDNA encodes a 490 aa protein since a second AUG codon, in an equally favorable initiator context, is present 9 codons downstream from the first AUG. Regardless, an aspect of the instant invention is the 1497-bp cDNA whether it encodes a 499- aa or 490-aa protein. This cDNA is on deposit with the American Type Culture Collection under accession number 98169.

## Example 2

### Properties of Cell Growth Regulatory Protein

The Cell Growth Regulatory Protein described in Example 1, and encoded by the cDNA on deposit with the American Type Culture Collection under accession number 98169, was tested for kinase activity by its capacity to inactivate Cdk/cyclin complexes in a cell-free extract system. The baculovirus expression system described in Example 3 was used to produce insect cell extracts that contained high levels of human Cdk, cyclin, Wee1, and the Cell Growth Regulatory Protein. The production of these proteins are either known in the art, or described in the following Examples. It is known previously that Cdk/cyclin kinase activation can be reconstituted upon mixing individual Cdk and cyclin-containing lysates (Desai et al. (1992) Mol.Biol. Cell 571-582); thus, if the Cell Growth Regulatory Protein encodes a kinase that inhibits the activity of the Cdk/cyclin complex, this would be readily detected by performing the appropriate phosphorylation assays.

We first assessed the ability of this assay to detect Wee1 activity. Wee1 is a kinase that is known to phosphorylate Cdc2/CyclinB1, but it phosphorylates only one residue on Cdc2, and that is Tyrosine-15. Thus, experiments were conducted to compare and contrast its phosphorylation pattern of Cdc2 to that produced by the Cell Growth Regulatory Protein.

Lysates containing wild-type Cdc2 were mixed with Cyclin B1 lysates and with either mock-infected or Wee1-containing lysates. After a brief incubation, an aliquot of this lysate mixture was removed and Cdc2/CyclinB1 kinase activity was determined by performing a histone H1 kinase reaction, as described by Booher et al. (1993) EMBO J. vol. 12: 3417-3426. It was observed that the addition of Wee1 lysates effectively inhibited Cdc2/CyclinB1 kinase activation. Also, the Wee1 inhibitory activity was dependent on the presence of the phosphorylatable Tyrosine-15 residue in Cdc2, since the mutant constructs, Cdc2-F15/CyclinB1 and Cdc2-AF/CylinB1 complexes retained full activity in the presence of Wee1 lysates.

When Cell Growth Regulatory Protein lysates were combined together with wild-type Cdc2 and CyclinB1-containing lysates, a dramatic reduction in Cdc2 kinase activation was observed, similar to that observed for Wee1. Furthermore, such lysates also inhibited both Cdc2-A14 and Cdc2-F15 activation indicating that the Cell Growth

Regulatory Protein can phosphorylate either Threonine 14 or Tyrosine 15 inhibitory sites in Cdc2; though, Cell Growth Regulatory Protein preferentially phosphorylates Threonine 14 since Cdc2-A14 lysates consistently retain some histone H1 kinase activity. Immunoblotting of the Cell Growth Regulatory Protein/Cdc2/CyclinB1 mixed lysates, using phosphotyrosine antibodies as probes (Wong et al. (1992) Cell vol. 69: 551-558, and New England Biolabs Catalogue) showed that Cdc2 was phosphorylated on Tyrosine 15 to some extent.

Human Wee1 is capable of phosphorylating Cdk2/cyclin A and Cdk2/cyclin E complexes in vitro. To determine whether the Cell Growth Regulatory Protein can also inactivate the Cdk2/cyclin complex, the histone H1 kinase in Cdk2 lysates that had been activated in the presence of Cell Growth Regulatory Protein containing lysates was measured. A Cdk2-AF mutant was used as a reference to control for any non-Threonine 14 or Tyrosine 15 phosphorylation inhibitory activity that may be present in the Cell Growth Regulatory Protein lysate. The results showed an equivalent amount of histone H1 kinase activity was present in wild-type Cdk2 and Cdk2-AF lysates that had been mixed with either cyclin A or cyclin E lysates in the presence of Cell Growth Regulatory Protein, indicating that neither Cdk2/cyclin A nor Cdk2/cyclin E complexes are negatively regulated by the Cell Growth Regulatory Protein.

Additional experiments were done to determine if the Cell Growth Regulatory Protein would inhibit Cdc2/cyclin A activation. Analysis of a Cell Growth Regulatory Protein/Cdc2/cyclin A mixed lysate revealed that it had greatly reduced histone H1 kinase activity compared to a mock-treated or Cdc2-AF control lysate. Thus, Cell Growth Regulatory Protein can readily inhibit Cdc2/cyclin A activation. A simple interpretation of these results is that the Cdk subunit, not the cyclin subunit, is a key determinant for whether a particular Cdk/cyclin complex is recognized by the Cell Growth Regulatory Protein kinase.

Additional experiments were conducted on the kinase substrate specificity by testing whether Cell Growth Regulatory Protein could phosphorylate directly various Cdk/cyclin complexes. For these experiments, various combinations of affinity purified Cell Growth Regulatory Protein, Wee1, and Cdk/cyclin complexes were incubated together in a kinase reaction containing [ $\gamma$ - $^{32}$ P]ATP followed by SDS-PAGE analysis of the  $^{32}$ P-labeled proteins. These experiments showed that both

purified Cell Growth Regulatory Protein and Wee1 kinases were capable of phosphorylating wild-type Cdc2 as well as a kinase-deficient Cdc2 mutant (K-), each of which was complexed with cyclin B1. It was further determined that the Cdc2-AF mutant was not phosphorylated by Cell Growth Regulatory Protein or Wee1, indicating that these kinases were phosphorylating either Cdc2 residue Threonine 14, or Tyrosine15, or both. A low level of  $^{32}\text{P}$ -labeled Cdc2 was also detected in the untreated wild-type Cdc2/cyclin B1 kinase reaction. The basis of this Cdc2 phosphorylation is unknown but a similar observation has been reported by Watanabe et al. (1995) EMBO J. vol. 14: 1878-1891. It should also be noted that we used a truncated Wee1 kinase in these studies because an unidentified 40 kD protein, which became highly  $^{32}\text{P}$ -labeled in the Cdc2/cyclin B1 kinase reaction, co-purified with full-length Wee1 during affinity chromatography from Sf9 cell lysates.

Further experiments showed that when Cdk2/cyclin complexes were used as substrates in these kinase reactions, Cell Growth Regulatory Protein failed to phosphorylate Cdk2 that was complexed with either cyclin A or cyclin E. In contrast, Wee1 readily phosphorylated Cdk2 that was complexed with either cyclin A and cyclin E, which is consistent with the findings of Watanabe et al. (1995) EMBO J. vol. 14: 1878-1891. We also tested a Cdk4/cyclin D1 complex and found that neither Cell Growth Regulatory Protein nor Wee1 phosphorylated the Cdk4 subunit.

Thus, the above results establish that using either a crude lysate system or an in-vitro kinase reaction using purified components, it was consistently observed that Cell Growth Regulatory Protein only phosphorylated and inactivated Cdk/cyclin complexes in which Cdc2 was the catalytic subunit.

Because the above experiments with Cell Growth Regulatory Protein were done on Cdc2 complexed to cyclins, experiments were conducted to assess whether Cell Growth Regulatory Protein phosphorylation of Cdc2 requires cyclin association. Thus, an experiment was performed with Cell Growth Regulatory Protein and monomeric Cdc2 as substrate. The results showed that Cell Growth Regulatory Protein did not phosphorylate monomeric Cdc2. However, reconstitution of the Cdc2/cyclin B1 complex, by pre-incubating monomeric Cdc2 with monomeric cyclin B1, enabled Cell Growth Regulatory Protein to phosphorylate Cdc2, indicating that Cell Growth Regulatory Protein can only phosphorylate Cdc2 that is complexed with a cyclin.

subunit. For comparison, similar experiments were conducted with the truncated Wee1 kinase, and it was observed that it was capable of  $^{32}\text{P}$ -labeling monomeric Cdc2, but this phosphorylation was enhanced when the cyclin-bound form was reconstituted, which is consistent with previous studies. See, Parker et al. (1995) PNAS vol. 92: 9638-9642.

5 Because Cell Growth Regulatory Protein failed to phosphorylate Cdc2-AF complexed with cyclin B1, it must phosphorylate wild-type Cdc2 on either Threonine 14 or Tyrosine 15, or both. Phosphoamino acid analysis of Cdc2 that had been  $^{32}\text{P}$ -labeled by Cell Growth Regulatory Protein revealed that phosphorylation occurred only on threonine residues, indicating that Cdc2-Threonine14 was the sole site of Cell Growth  
10 Regulatory Protein phosphorylation. The absence of phosphotyrosine is unlikely due to the use of the Cdc2(K-) mutant since the Tyrosine 15 residue was readily phosphorylated by full-length Wee1. Further phosphoamino analysis revealed that Cell Growth Regulatory Protein autophosphorylated itself on tyrosine, threonine, and serine residues, while full-length Wee1 only contained phosphotyrosine.

### 15 Example 3

#### Expression of Cell Growth Regulatory Proteins

The Cell Growth Regulatory Proteins were generally expressed in and purified from baculovirus Sf9 infected cells. Methods for expressing proteins in baculovirus, as well as growing Sf9 cells are well known in the art, and detailed procedures can be  
20 found in M. Summers and G. Smith in "A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures," Texas Agricultural Experiment Station, Bulletin No. 1555 (May, 1987 or in EPS 127,839 to G. E. Smith and M. D. Summers. Depending on the protein expressed, a preferred construct was generated and used to express the protein in Sf9 cells. These are as follows.

25 **Cell Growth Regulatory Protein Baculovirus Expression Vector:** A Cell Growth Regulatory Protein-containing DNA fragment corresponding to base-pairs 354-1925 (numbering according to Figure 1) was PCR amplified using cDNA clone 16 as template and the 5' primer 5'-TCGAATTCATGCCCATGCCCACG-3', corresponding to bases 353-368 bases and the 3' primer 5'-CAGAGAAGACCATGGAGTTCC-3', corresponding to the reverse  
30 complement of bases 1904-1925; and introduced EcoRI restriction site within the 5' primer is italicized. This PCR fragment was cloned by T-cloning into vector pT7Blue (Novagen). An EcoRI-XbaI Cell Growth Regulatory Protein-containing DNA fragment was isolated from this



vector and inserted into the EcoRI and XbaI sites of the baculovirus transfer vector pAcO-G1. This vector was generated from pAcOG, a derivative of pAcC13 (See, Figure 3, and Rubinfeld, B., et al. Cell 65, 1033-1042 (1991)) in which the polylinker in pAcC13 was replaced with a synthetic linker engineered to encode an initiating methionine, the Glu-Glu (See, Grussenmyer, T., et al. Proc. Natl. Acad. Sci. U.S.A. 82, 7952 (1985)) epitope tag, and a multiple cloning site containing several stop codons (See, Rubinfeld, B., et al. J. Biol. Chem, 270, 5549-5555 (1995)). The baculovirus expression vector, pAcO-G1, expresses a truncated Cell Growth Regulatory Protein hybrid protein (residues 1-9 deleted) that is fused with an N-terminal 12-residue peptide (MEYMPMEGTRPE).

The Glu-Glu epitope tag facilitates purification of the Cell Growth Regulatory Protein fragment by antibody affinity as described below in Example 4.

**Wee1 Baculovirus Expression Vector:** The full-length human Wee1 coding region, corresponding to base-pairs 254-2194 (numbering according to Watanabe et al. (1995) EMBO J. VOL. 14: 1878-1891), was isolated from the Wee1-containing clone 1E-12 [Watanabe, et al] and inserted into the baculovirus transfer vector pAcO-G1. This baculovirus expression vector expresses Wee1 protein that is fused with an N-terminal 23-residue peptide (MEYMPMEGTRPEFELGTRGSDWS) that contains the Glu-Glu epitope tag. Additionally, Wee1 residue Serine 2 was changed to glycine. A baculovirus expression vector expressing a truncated Wee1 protein was constructed by inserting a Wee1-containing DNA fragment corresponding to base-pairs 896-2194 (numbering according to Watanabe et al.) into pAcO-G3 (Figure 3). This recombinant Wee1 protein is fused with an N-terminal 17-residue peptide (MEYMPMEEVTPGLNSAWS) that contains the Glu-Glu epitope tag.

**Cdk Baculovirus Expression Vector:** Baculovirus expression vectors expressing C-terminal influenza hemagglutinin (HA)-tagged Cdc2-wt, F15, AF, and K- proteins, and C-terminal HA-tagged Cdk2-wt and AF proteins were all provided by Dr. David Morgan (UCSF) and have been previously described [Desai et al (1992) Mol Biol Cell 3:571-582; Gu et al. (1992) EMBO J., 11:3995-4005]. It may be noted that F15, AF, and K- refers to mutants of Cdc2. In some cases the HA-tag was replaced with a 12-residue peptide, TMEYMPMEGYQA for Cdc2 and SMEYMPMEGYQA for Cdk2, by inserting the respective coding regions into the baculovirus expression vector pAcO-G2 (Figure

3). The Cdc2-A14 mutation (Threonine 14 to Alanine) was created by site-directed oligo mutagenesis.

**Cyclin Baculovirus Expression Vector:** The human cyclinA coding region was inserted into baculovirus expression vector pAcO-G3 such that the expressed cyclinA protein is fused at the N-terminus with a 20-residue peptide (MEYMPMEEVPGLNSCSPGAV). The human cyclin A coding region is described by Pines and Hunter (1990) Nature, vol. 346: 760-763. A PstI-SacI DNA fragment containing the entire CyclinA coding region was cloned into PstI/SacI-cut baculovirus transfer vector pAcO-G3. The human cyclinB coding region was also inserted into pAcO-G3 and this expresses cyclinB1 protein that contains an N-terminal 20-residue peptide (MEYMPMEEVPGLNSCRARIS). The cyclinB1 coding region is described by Pines and Hunter (1989) Cell, vol. 58: 833-846. The coding region is contained in a 1.5 kb DNA fragment which was cloned into BamHI/NotI cut baculovirus transfer vector pAcO-G3. The human cyclinE coding region was cloned into pAcO-G1 and was expressed with an N-terminal 21-residue peptide (MEYMPMEGTRPEFRDAKERDT). The CyclinE coding region is described by Koff et al. (1991) Cell, vol. 66: 1217-1228. An EcoRI-EcoRV DNA fragment containing the entire Cyclin E coding region was cloned into EcoRI/SmaI cut baculovirus transfer vector pAcO-G1. Each of these N-terminal peptides contain the Glu-Glu epitope. The GST-cyclinB1 baculovirus expression vector expresses cyclinB1 with an N-terminal glutathione S-transferase fusion.

#### Example 4

##### Purification of Cell Growth Regulatory Protein

Large-scale production of heterologous proteins in Sf9 cells with recombinant baculoviruses and subsequent purification of Glu-Glu-tagged proteins was performed as follows. The Cdk/cyclin complexes were activated by mixing separate Cdk and cyclin-containing Sf9 extracts together with CAK (Cdk7/cyclin H; see, Fisher and Morgan (1994) Cell, vol. 78: 713-724) and ATP prior to purification. Briefly, baculovirus containing the appropriate construct was produced by transfecting the above described plasmids into SF9 cells, and isolating the corresponding baculovirus using essentially the methods described in Pharmingen's cat. no. 21100D, BaculoGoldtm/Baculovirus DNA. Virus was isolated from individual plaques, and

used to infect Sf9 cells. The cells were grown for 4 days, isolated by centrifugation, and cell extracts made by solubilizing the cell pellet. Briefly, recombinant Sf9 cells were pelleted, lysed in 5 volumes of [20mM Tris (pH8.0), 1mM EDTA, 10mg/ml each of leupeptin, pepstatin, pefabloc, 1mM aprotinin and 1mM DTT] and incubated on ice for 5 10 minutes. NaCl was then added to a final concentration of 150mM, incubated at room temperature for 10 minutes and centrifuged. The resulting supernatant was loaded onto a 1-ml affinity column containing a mouse Glu-Glu monoclonal antibody covalently cross-linked to protein G-Sepharose. See, Grussenmyer, T., et al., Proc. Natl. Acad. Sci. U. S. A. vol. 82, pp. 7952-7954 (1985). The column was washed with 10-10 15ml of lysis buffer and eluted with 100mg of Glu-Glu peptide (EYMPME) per ml in the same buffer. Fractions were collected and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the peak fractions were pooled and based on purity subjected to further purification on HPLC columns which include Resource Q, Resource S and Resource Eth (Pharmacia). For purification of insoluble 15 proteins, in particular 091-21A31, Sequence ID No. 1, recombinant Sf9 cells were pelleted, lysed in 5 volumes of [20mM Tris (pH8.0), 137mM NaCl, 1mM EGTA, 1.5mM MgCl<sub>2</sub>, 2%SDS, 10mg/ml each of leupeptin, pepstatin, pefabloc, 1mM aprotinin and 1mM DTT], incubated at room temperature for 20-30 minutes and ultra centrifuged. The upper phase was removed, NaCl was adjusted to 400mM and recentrifuged. The 20 clarified supernatant was then diluted 1:10 in 1 X TG buffer [20mM Tris (pH8.0), 137mM NaCl, 1mM EGTA, 1.5mM MgCl<sub>2</sub>, 1% Triton X100, 10% glycerol, 10mg/ml each of leupeptin, pepstatin, pefabloc, 1mM aprotinin and 1mM DTT], filtered through a 3uM Gelman Versapore filter and loaded onto a 1-ml anti-Glu-Glu affinity column. See, Rubinfeld, B., et al., Mol. Cell. Bio. 12, 4634-4642 (1992). The column was washed with 25 10-15ml of 1 X TG buffer with 400mM NaCl and eluted in 1 X TG buffer with 1% SDS and 100mg/ml Glu-Glu peptide. Fractions were analyzed by SDS-PAGE.

#### Example 5

#### Preparation of Antibody to Cell Growth

#### Regulatory Protein

30 Polyclonal rabbit antibodies were raised against three different Cell Growth Regulatory Protein specific peptides. Each of the peptides was conjugated to maleimide activated KLH (Pierce) prior to immunization. Rabbits 12390 and 12391

were injected with peptide C-47 corresponding to amino acids 472-487 (CNSEPPRGSFPSFEPRN) near the C-terminus of Cell Growth Regulatory Protein. Rabbits 12392 and 12393 were injected with peptide C-49 corresponding to amino acids 486-499 (CRNLLSMFEDTLDPT) at the C-terminus of Cell Growth Regulatory Protein. Rabbits 12400 and 12401 were injected with peptide C-48 corresponding to amino acids 10-23 (MPMPTEGTPPPLSGC) near the N-terminus of Cell Growth Regulatory Protein. The Cell Growth Regulatory Protein peptides were synthesized by Multiple Peptide Systems and the rabbit polyclonal antibodies were generated at Pocono Rabbit Farm and Laboratory, Inc. These antibodies were used to determine the cellular location of Cell Growth Regulatory Protein, as described in Example 7.

### Example 6

#### Cell Growth Regulatory Proteins that lack a Trans-Membrane Region

The Cell Growth Regulatory Protein encoded by the cDNA sequence shown in Example 1 (American Type Culture Collection, Accession Number 98169) contains a predicted 20 amino acid transmembrane segment just C-terminal of the kinase catalytic domain (Mueller et al (1995) Science 270, pp 86-90). To determine if this segment directs this Cell Growth Regulatory Protein to membranes, we expressed a series of Cell Growth Regulatory Protein deletion mutants in mammalian cells and assessed their ability to associate with the membrane fraction by immunoblotting. Four truncated Cell Growth Regulatory Protein mutants were constructed and included deletions of the N-terminus, C-terminus, and the transmembrane segments. These constructs are shown schematically in Figure 2. The expressed Cell Growth Regulatory Protein forms include Cell Growth Regulatory Protein-1b (residues 9-499), Cell Growth Regulatory Protein-1A (residues 58-499), Cell Growth Regulatory Protein-4b (residues 9-292), and Cell Growth Regulatory Protein-ΔT.M. (residues 9-499 with residues 379-398 deleted). They were generated as follows.

Protein-1b, A Cell Growth Regulatory Protein-containing DNA fragment (bases 353-1925, numbering according to Figure 1) was PCR-amplified using cDNA clone 16 as template and the 5' primer 5'-TCGGATCCATGCCCATGCCACG-3', corresponding to bases 353-368 bases and the 3' primer 5'-CAGAGAAGACCATGGAGTTCC-3',

corresponding to the reverse complement of bases 1904-1925; an introduced BamHI restriction site within the 5' primer is italicized. This PCR fragment was cloned by T-cloning into vector pT7Blue (Novagen). An EcoRI-XbaI Cell Growth Regulatory Protein-containing DNA fragment was isolated from this vector and inserted into the

5 EcoRI and XbaI sites of the baculovirus transfer vector pCAN-HA1. Protein-1A, an EcoRI DNA fragment containing the Cell Growth Regulatory Protein corresponding to base-pairs 547-1976 (obtained from a partial cDNA clone in vector pZAPII) was cloned into the EcoRI site of vector pCAN-HA1. This clone contains approximately 20 additional base-pairs within the 3' non-coding region. Protein-4b, a Cell Growth

10 Regulatory Protein-containing DNA fragment (bases 353-1355, numbering according to Figure 1) was PCR-amplified using cDNA clone 16 as template and the 5' primer 5'-TCGGATCCATGCCCATGCCACG-3', corresponding to bases 353-368 bases and the 3' primer 5'-CAGAGAAGACCATGGAGTTCC-3', corresponding to the reverse complement of bases 1340-1355; and introduced BamHI restriction site within the 5'

15 primer is italicized. This PCR fragment was cloned by T-cloning into vector pT7 Blue (Novagen). An EcoRI-XbaI Cell Growth Regulatory Protein-containing DNA fragment was isolated from this vector and inserted into the EcoRI and XbaI sites of the baculovirus transfer vector pCAN-HA1. Protein-A.T.M., a BamHI DNA fragment containing the analogous Protein-1b coding region, except that base-pairs 1462-1518

20 were deleted by oligo mutagenesis, was cloned into the BamHI site of vector pCAN-HA1.

As noted, each truncated form was fused at the N-terminus with the hemagglutinin (HA) epitope tag (See, Field et al. (1988) Mol. Cell. Biol., vol. 8: 2159-2165) thus permitting specific detection of the transiently expressed gene product by

25 probing immunoblots with the Mab 12CA5 which recognizes the hemagglutinin epitope tag (See, Wilson et al. (1984) Cell, vol. 37: 767-778). These plasmids were transiently transfected into human C33A cells using standard techniques, followed by immunoblot analysis of the soluble and membrane fractions. Briefly, transient transfections of C33A cells were done on cells grown in DME, supplemented with fetal

30 calf serum and 10% BSA, using the calcium phosphate precipitation DNA transfection protocol. The S100 and P100 fractions (100,000g supernatant and pellet) were prepared from cells (48 hr post-transfection) that had been lysed by dounce homogenization in an

ice-chilled hypotonic buffer (10 mM Hepes, pH 7.5, 5 mM KCl, 1.5 mM MgCl<sub>2</sub>, protease inhibitors). The membrane-bound proteins in the P100 fractions were solubilized by extracting the P100 pellets with 1% NP40.

The results showed that both of the N-terminal truncation mutants (-1b and -1A) were present solely in the membrane fraction whereas the two C-terminal mutants (-4b, and -ΔT.M.) were present in the cytosol. These transiently expressed proteins comigrated exactly with the analogous proteins expressed in a coupled transcription/translation system. These results demonstrate that the human Cell Growth Regulatory Protein localizes to membranes via the C-terminal 20-aa transmembrane segment.

Moreover, additional experiments established that the Cell Growth Regulatory Proteins -1b and ΔT. M. exhibit kinase activity. Thus, these constructs will have utility in the assays described below that are aimed at identifying compounds with prophylactic or therapeutic activity for treating diseases arising from unwanted cell growth.

#### Example 7

##### Phosphorylated Cell Growth Regulatory Protein

Experiments were conducted to show that the Cell Growth Regulatory Protein encoded by the cDNA sequence described in Example 1, and on deposit with the American Type Culture Collection, Accession Number 98169, is phosphorylated and that phosphorylation is a function of the cell cycle.

The experiments were conducted using Cell Growth Regulatory Protein in lysates prepared from human CEM cells that had been arrested at G1, S and M phases of the cell cycle by treatment with mimesine, hydroxyurea, and nocadazole, respectively. Synchronization of cells by drug block arrests was performed by culturing CEM cells for 16-23 hr in the presence of either 5 mM hydroxyurea (Calbiochem), 1 mM mimosine (Sigma), or 50 ng/ml nocadazole (Calbiochem). Immunoblot analysis was performed using antibody from rabbit 12390 that was injected with peptide C-47 corresponding to amino acids 472-487 (CNSEPPRGSFPSFEPRN) near the C-terminus of Cell Growth Regulatory Protein. The results revealed that Cell Growth Regulatory Protein in mitotic lysates shifted to a slower migrating form. The main contribution to this modification is phosphorylation, since phosphatase treatment of the mitotic lysate

decreased the migration of Cell Growth Regulatory Protein to nearly that observed in G1 and S-phase extracts. At least five ladderling Cell Growth Regulatory Protein forms were detectable in the dilute phosphatase-treated mitotic lysate, indicating that multiple Cell Growth Regulatory Protein residues are phosphorylated during mitosis.

- 5 Additionally, immunoblot analysis of cytosolic and detergent-extracted P100 fractions prepared from nocodazole-arrested CEM cells revealed that the phosphorylated forms of Cell Growth Regulatory Protein remained membrane-associated.

### Example 8

#### Assays for the Identification of Drugs to treat

10

#### Hyperproliferative Cell Growth Diseases

A key aspect of the instant invention is the presentation of assays that permit the identification of unique compounds that can be used to control unwanted, or hyperproliferative cell growth. Such compounds will have significant applications for treating a variety of diseases that involve unwanted cell growth including cancer, restenosis and others known to the skilled practitioner of this art.

15

Two assays are described below. Both assays rely on inhibiting the activity of Cell Growth Regulatory Protein which in turn prevents the phosphorylation of cdc2/cyclinB1, which further, in turn, causes cells to progress through the cell cycle without the necessary control mechanisms. This has the effect of killing the constantly cycling cells. Thus, the assays described below measure the phosphorylation of a suitable Cell Growth Regulatory Protein substrate in the presence of test compounds to determine their effect on Cell Growth Regulatory Protein kinase activity.

20

##### 1. Cdc2/cyclinB1 histone H1 kinase activity.

The first assay indirectly measures the kinase activity of Cell Growth Regulatory Protein. Cell Growth Regulatory Protein phosphorylates Cdc2/cyclinB1, resulting in the inactivation of the Cdc2/cyclinB1 kinase activity, as measured by the phosphorylation of histone H1. Compounds that inactivate Cell Growth Regulatory Protein kinase activity thus will cause Cdc2/cyclinB1 to retain kinase activity and can be detected by increased <sup>32</sup>P-labeling of histone H1. Other Cdc2/cyclinB1 substrates can also be used, including GST-Rb, Cdc25, or peptides containing Cdc2 phosphorylation sites. Alternatively, other indirect measures of Cdc2/cyclinB1 activity could be incorporated into this assay, including <sup>32</sup>P-labeling of the cyclinB1

30

subunit or the reduced electrophoretic mobility of Cdc2 by PAGE. Other Cdk/cyclin complexes, such as Cdc2/cyclinA or Cdc2/cyclinB2, may be used in this in-vitro Cell Growth Regulatory Protein kinase reaction. This Cell Growth Regulatory Protein assay can be performed utilizing purified Cell Growth  
5 Regulatory Protein and Cdc2/cyclinB1 proteins as well as utilizing a crude Sf9 lysate mixing format.

For example, frozen pellets of Sf9 cells expressing appropriate recombinant proteins are thawed and lysed by dounce homogenization in 10 mM Hepes, pH 7.5, 1 mM EDTA, 1% NP40, 1 mM DTT and protease inhibitors [1 mM Pefabloc, 5  
10 mg/ml leupeptin, 5 mg/ml leupeptin] at 4°C, followed by ultracentrifugation for 30 min at 42 kpm in a TLA45 rotor at 4°C. The clarified lysates are aliquoted and stored at -80°C. Activation of Cdc2/cyclinB complexes are performed by mixing combinations of Cdc2 and cyclinB lysates, with Cell Growth Regulatory Protein lysates, together with an ATP regeneration system (10X stock: 0.5 mg/ml creatine  
15 phosphokinase, 10 mM ATP, 350 mM phosphocreatine, 1 mM, 2 mM Hepes, pH 7.5) at 25°C for 25 min. Additionally, compounds that are tested for Cell Growth Regulatory Protein inhibitory activity are added to the reaction mixture over a range of concentrations, preferably from nanomolar to millimolar. Next, to determine the effect of a compound on Cell Growth Regulatory Protein activity by its effect on  
20 Cdc2/cyclinB kinase activity, the latter is measured using 1 ul of lysate mixture incubated with 9 ul of a histone H1 kinase mix [50 mM Tris, pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 5 ug histone H1, 50 uM ATP, 1 uCi [<sup>32</sup>P]ATP (3000Ci/mmol)] for 10-15 min at 25°C. The amount of histone H1 phosphorylation is measured using standard polyacrylamide gel electrophoresis and autoradiography methods.  
25 The appropriate controls are run to ensure that the compounds being tested are affecting Cell Growth Regulatory Protein activity, and not interacting directly with cdc2/cyclinB.

Those compounds identified to affect Cell Growth Regulatory Protein activity will have significant prophylactic and therapeutic benefit for the treatment of  
30 hyperproliferative diseases.



## 2. Inhibition of Cell Growth Regulatory Protein substrate phosphorylation.

Compounds are identified that inactivate Cell Growth Regulatory Protein kinase activity by directly measuring the phosphorylation of Cell Growth Regulatory Protein substrates, preferably Cdc2 complexed with cyclinB1. The assay is run using, as described above, lysates of the appropriate proteins, and under suitable reaction conditions including [ $\gamma$ - $^{32}$ P]ATP in the in-vitro kinase reaction mixture. Alternatively, phospho-specific antibodies (New England Biolabs) such as an anti-phosphothreonine antibody, may be used to detect phosphorylation of Cell Growth Regulatory Protein substrates. Additionally, the assay may be run using purified protein components.

Compounds that inhibit the kinase activity of Cell Growth Regulatory Protein will prevent the phosphorylation of cdc2/cyclinB1. Such compounds will have significant prophylactic and therapeutic benefit for the treatment of hyperproliferative diseases.

### DEPOSIT OF CLONES

The following microorganisms or clones were deposited with the American Type Culture Collection (ATCC), Rockville, Maryland, on the dates indicated and were assigned the indicated accession number:

<u>Clone</u>	<u>Access. No.</u>	<u>Date of Deposit</u>
pBSK-myt1	98169	September 17, 1996

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- 5 (i) APPLICANT: Robert M. Booher  
Ali Fattaey
- (ii) TITLE OF INVENTION: Identification of Human cDNA Encoding a  
Putative Myt1 Kinase Homolog
- (iii) NUMBER OF SEQUENCES: 2
- 10 (iv) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: GNYX Pharmaceuticals, Inc.
- (B) STREET: 3031 Research Drive
- (C) CITY: Richmond
- (D) STATE: CA
- 15 (E) COUNTRY: USA
- (F) ZIP: 94806
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- 20 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER: US Unknown
- (B) FILING DATE:
- 25 (C) CLASSIFICATION:
- (vii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Giotta, Gregory
- (B) REGISTRATION NUMBER: 32,028
- (C) REFERENCE/DOCKET NUMBER: GNYX1025
- 30 (ix) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: (510) 262-8710
- (B) TELEFAX: (510) 222-9758

## (2) INFORMATION FOR SEQ ID NO:1:

- 35 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1976 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- 40 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 327..1823

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

5	CAGGACTCCG GTGAGGGGGA ACGGCCCGTG AACGGGCGCG GAGCTGCTCG CGTCCCGCCC	60
	AGTGGCCCCA GGGCTTCCCG ACACCCACGG AGTGAAGTCA GGTGGGCCCC TGCCTGGGAG	120
	GAACCTACCG TCTACCGGGA AAGSTGGCCA GCAGATGTGT CGGGCTGSGT GAGAGGGTGA	180
	GGGAGAGCGG CCGGATCCCG CAGGGCCCCG GAAGCTGCGG AGTCAACCCG CGCCTGGCCT	240
	TAGCTCAGGG ACACCCCTGA TTAAGSTGG AGCTCTGTGT CGTGGCTCCG CGGTCCCACC	300
10	ACTGAAGCTG TTGGGCCAGG CCAGTC ATG CTA GAA GGG CCT CCG GCA CTG GCC	353
	Met Leu Glu Arg Pro Pro Ala Leu Ala	
	1 5	
	ATG CCC ATG CCC ACG GAG GGC ACC CCG CCA CCT CTG AGT GGC ACC CCC	401
	Met Pro Met Pro Thr Glu Gly Thr Pro Pro Pro Leu Ser Gly Thr Pro	
15	10 15 20 25	
	ATC CCA GTC CCA GCG TAC TTC CGC CAC GCA GAA CCT GGA TTC TCC CTC	449
	Ile Pro Val Pro Ala Tyr Phe Arg His Ala Glu Pro Gly Phe Ser Leu	
	30 35 40	
	AAG AGG CCC AGG GGG CTC AGG CGG AGC CTC CCA CCT CGG GCG CCT GCG	497
20	Lys Arg Pro Arg Gly Leu Ser Arg Ser Leu Pro Pro Pro Pro Pro Ala	
	45 50 55	
	AAG GGC AGC ATT CCC ATC AGC CGC CTC TTC CCT CCT CGG ACC CCA GGC	545
	Lys Gly Ser Ile Pro Ile Ser Arg Leu Phe Pro Pro Arg Thr Pro Gly	
	60 65 70	
25	TGG CAC CAG CTG CAG CCC CGG CGG GTG TCA TTC CGG GCG CAG GCC TCA	593
	Trp His Gln Leu Gln Pro Arg Arg Val Ser Phe Arg Gly Glu Ala Ser	
	75 80 85	
	GAG ACT CTG CAG AGC CCT GGG TAT GAC CCA AGC CGG CCA GAG TCC TTC	641
	Glu Thr Leu Gln Ser Pro Gly Tyr Asp Pro Ser Arg Pro Glu Ser Phe	
30	90 95 100 105	
	TTC CAG CAG AGC TTC CAG AGG CTC AGC CGC CTG GGC CAT GGC TCC TAC	689
	Phe Gln Gln Ser Phe Gln Arg Leu Ser Arg Leu Gly His Gly Ser Tyr	
	110 115 120	
	GGA GAG GTC TTC AAG GTG CGC TCC AAG GAG GAG GCG CGG CTC TAT CGC	737
35	Gly Glu Val Phe Lys Val Arg Ser Lys Glu Asp Gly Arg Leu Tyr Ala	
	125 130 135	
	GTA AAG CGT TCC ATG TCA CCA TTC CGG GGT CTA AAG GAT CGG GCG CGC	785
	Val Lys Arg Ser Met Ser Pro Phe Arg Gly Pro Lys Asp Arg Ala Arg	
	140 145 150	
40	AAG TTG GCG GAG GTG GCG ACC CAC GAG AAG TTC Phe GAG CAG CCA TCC	833
	Lys Leu Ala Glu Val Gly Ser His Glu Lys Pro Gly Gln His Pro Cys	
	155 160	

	TGC GTG GGG CTG GAG CAG GGC TGG GAG GAG GGC GGC ATC CTG TAC CTG	881
	Cys Val Arg Leu Gln Gln Ala Trp Gln Gln Gly Gly Ile Leu Tyr Leu	
	170 175 180 185	
	CAG ACG GAG CTG TGC GGG CCC AGC CTG CAG CAA CAC TGT GAA GGC TGG	929
5	Gln Thr Glu Leu Cys Gly Pro Ser Leu Gln Gln His Cys Glu Ala Trp	
	190 195 200	
	GGT GGC AGC CTG CCT GAG GGC CAG GTC TGG GGC TAC CTG GGG GAC ACG	977
	Gly Ala Ser Leu Pro Gln Ala Gln Val Trp Gly Tyr Leu Arg Asp Thr	
	205 210 215	
10	CTG CTT GCC CTG GCC CAT CTG CAC AGC CAG GGC CTG GTG CAC CTT GAT	1025
	Leu Leu Ala Leu Ala His Leu His Ser Gln Gly Leu Val His Leu Asp	
	220 225 230	
	GTC AAG CCT GGC AAC ATC TTC CTG GGG CCC GGG GGC CCC TGC AAG CTG	1073
	Val Lys Pro Ala Asn Ile Phe Leu Gly Pro Arg Gly Arg Cys Lys Leu	
15	235 240 245	
	GGT GAC TTC GGA CTG CTG GTG GAG CTG GGT ACA GCA GGA GCT GGT GAG	1121
	Gly Asp Phe Gly Leu Leu Val Glu Leu Gly Thr Ala Gly Ala Gly Glu	
	250 255 260 265	
	GTC CAG GAG GGA GAC CCC GGC TAC ATG GGC CCC GAG CTG CTG CAG GGC	1169
20	Val Gln Glu Gly Asp Pro Arg Tyr Met Ala Pro Glu Leu Leu Gln Gly	
	270 275 280	
	TCC TAT GGG ACA GCA GCG CAT GTG TTC AGT CTG GGC CTC ACC ATC CTG	1217
	Ser Tyr Gly Thr Ala Ala Asp Val Phe Ser Leu Gly Leu Thr Ile Leu	
	285 290 295	
25	GAA GTG GCA TGC AAC ATG GAG CTG CCC CAC GGT GGG GAG GGC TGG CAG	1265
	Glu Val Ala Cys Asn Met Glu Leu Pro His Gly Gly Glu Gly Trp Gln	
	300 305 310	
	CAG CTG GGC CAG GGC TAC CTG CCC CCT GAG TTC ACT GCC GGT CTG TCT	1313
	Gln Leu Arg Gln Gly Tyr Leu Pro Pro Glu Phe Thr Ala Gly Leu Ser	
30	315 320 325	
	TCC GAG CTG CGT TCT GTC CTT GTC ATG ATG CTG GAG CCA GAC CCC AAG	1361
	Ser Glu Leu Arg Ser Val Leu Val Met Met Leu Glu Pro Asp Pro Lys	
	330 335 340 345	
	CTG GGG GGC ACG GGC GAG GGC CTG CTG GCA CTG CCT GTC TTG AGG CAG	1409
35	Leu Arg Ala Thr Ala Glu Ala Leu Leu Ala Leu Pro Val Leu Arg Gln	
	350 355 360	
	CCG GGG GGC TGG GGT GTG CTG TGG TGC ATG GGA GCG GAG GCC CTG AGC	1457
	Pro Arg Ala Trp Gly Val Leu Trp Cys Met Ala Ala Glu Ala Leu Ser	
	365 370 375	
40	CGA GGG TGG GGC CTG TGG CAG GGC CTG CTT TGT CTG CTG TGC TGG CTC	1505
	Arg Gly Trp Ala Leu Trp Gln Ala Leu Leu Ala Leu Leu Cys Trp Leu	
	380 385 390	

TGG CAT GGG CTG GCT CAC CCT GCC AGC TGG CTA CAG CCC CTG GGC CCG 1553  
 Trp His Gly Leu Ala His Pro Ala Ser Trp Leu Gln Pro Leu Gly Pro  
 395 400 405  
 CCA GCC ACC CCG CCT GAC TCA CCA CCC TGC AGT TTG CTC CTG GAC AGC 1601  
 5 Pro Ala Thr Pro Pro Asp Ser Pro Pro Cys Ser Leu Leu Leu Asp Ser  
 410 415 420 425  
 AGC TTC TCC ACC AAC TGG GAT GAC GAC AGC CTA GGG CCT TCA CTC TCC 1649  
 Ser Phe Ser Ser Asn Trp Asp Asp Asp Ser Leu Gly Pro Ser Leu Ser  
 430 435 440  
 10 CCT GAG GCT GTC CTG GGC CGG ACT GTG GGG AGC ACC TCC ACC CCC CGG 1697  
 Pro Glu Ala Val Leu Ala Arg Thr Val Gly Ser Thr Ser Thr Pro Arg  
 445 450 455  
 AGC AGG TGC ACA CCC AGG GAT GCC CTG GAC CTA ACT GAC ATC AAC TCA 1745  
 Ser Arg Cys Thr Pro Arg Asp Ala Leu Asp Leu Ser Asp Ile Asn Ser  
 15 460 465 470  
 GAG CCT CCT GGG GGC TCC TTC CCC TCC TTT GAG CCT CGG AAC CTC CTC 1793  
 Glu Pro Pro Arg Gly Ser Phe Pro Ser Phe Glu Pro Arg Asn Leu Leu  
 475 480 485  
 AGC ATG TTT GAG GAC ACC CTA GAC CCA ACC TGAGCCCCAG ATTCTGCCTC 1843  
 20 Ser Met Phe Glu Asp Thr Leu Asp Pro Thr  
 490 495  
 TGCACCTTTTA ACCTTTTATC CTGTCTCTCT CCGGTGCCCC TTGAAAGCTG GGCCCCCTCG 1903  
 GGAACTCCCA TGCTCTTCTC TGCTTGCCCG TGTCTAATAA AAAGTATTTG AACCTTGAAA 1963  
 AAAAAAAAAAG AAG 1976

25

(2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

30

- (A) LENGTH: 499 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:2:

35 Met Leu Gln Arg Pro Pro Ala Leu Ala Met Pro Met Pro Thr Gln Gly  
 1 5 10 15  
 Thr Pro Pro Pro Leu Ser Gly Thr Pro Ile Pro Val Pro Ala Tyr Phe  
 20 25 30  
 Arg His Ala Gln Pro Gly Phe Ser Leu Lys Arg Pro Arg Gly Leu Ser  
 35 40 45  
 40 Arg Ser Leu Pro Pro Pro Pro Pro Ala Lys Gly Ser Ile Pro Ile Ser  
 50 55 60  
 Arg Leu Phe Pro Pro Arg Thr Pro Gly Trp His Gln Leu Gln Pro Arg  
 65 70 75 80

Arg Val Ser Phe Arg Gly Glu Ala Ser Glu Thr Leu Gln Ser Pro Gly  
                     85                    90                    95  
 Tyr Asp Pro Ser Arg Pro Glu Ser Phe Phe Gln Gln Ser Phe Gln Arg  
                     100                    105                    110  
 5 Leu Ser Arg Leu Gly His Gly Ser Tyr Gly Glu Val Phe Lys Val Arg  
                     115                    120                    125  
 Ser Lys Glu Asp Gly Arg Leu Tyr Ala Val Lys Arg Ser Met Ser Pro  
                     130                    135                    140  
 Phe Arg Gly Pro Lys Asp Arg Ala Arg Lys Leu Ala Glu Val Gly Ser  
 10 145                    150                    155                    160  
 His Glu Lys Val Gly Gln His Pro Cys Cys Val Arg Leu Glu Gln Ala  
                     165                    170                    175  
 Trp Glu Glu Gly Gly Ile Leu Tyr Leu Gln Thr Glu Leu Cys Gly Pro  
                     180                    185                    190  
 15 Ser Leu Gln Gln His Cys Glu Ala Trp Gly Ala Ser Leu Pro Glu Ala  
                     195                    200                    205  
 Gln Val Trp Gly Tyr Leu Arg Asp Thr Leu Leu Ala Leu Ala His Leu  
                     210                    215                    220  
 His Ser Gln Gly Leu Val His Leu Asp Val Lys Pro Ala Asn Ile Phe  
 20 225                    230                    235                    240  
 Leu Gly Pro Arg Gly Arg Cys Lys Leu Gly Asp Phe Gly Leu Leu Val  
                     245                    250                    255  
 Glu Leu Gly Thr Ala Gly Ala Gly Glu Val Gln Glu Gly Asp Pro Arg  
                     260                    265                    270  
 25 Tyr Met Ala Pro Glu Leu Leu Gln Gly Ser Tyr Gly Thr Ala Ala Asp  
                     275                    280                    285  
 Val Phe Ser Leu Gly Leu Thr Ile Leu Glu Val Ala Cys Asn Met Glu  
                     290                    295                    300  
 Leu Pro His Gly Gly Glu Gly Trp Gln Gln Leu Arg Gln Gly Tyr Leu  
 30 305                    310                    315                    320  
 Pro Pro Glu Phe Thr Ala Gly Leu Ser Ser Glu Leu Arg Ser Val Leu  
                     325                    330                    335  
 Val Met Met Leu Glu Pro Asp Pro Lys Leu Arg Ala Thr Ala Glu Ala  
                     340                    345                    350  
 35 Leu Leu Ala Leu Pro Val Leu Arg Gln Pro Arg Ala Trp Gly Val Leu  
                     355                    360                    365  
 Trp Cys Met Ala Ala Glu Ala Leu Ser Arg Gly Trp Ala Leu Trp Gln  
                     370                    375                    380  
 Ala Leu Leu Ala Leu Leu Cys Trp Leu Trp His Gly Leu Ala His Pro  
 40 385                    390                    395                    400  
 Ala Ser Trp Leu Gln Pro Leu Gly Pro Pro Ala Thr Pro Pro Asp Ser  
                     405                    410                    415  
 Pro Pro Cys Ser Leu Leu Leu Asp Ser Ser Phe Ser Ser Asn Trp Asp  
                     420                    425                    430

Asp Asp Ser Leu Gly Pro Ser Leu Ser Pro Glu Ala Val Leu Ala Arg  
435 440 445  
Thr Val Gly Ser Thr Ser Thr Pro Arg Ser Arg Cys Thr Pro Arg Asp  
450 455 460  
5 Ala Leu Asp Leu Ser Asp Ile Asn Ser Glu Pro Pro Arg Gly Ser Phe  
465 470 475 480  
Pro Ser Phe Glu Pro Arg Asn Leu Leu Ser Met Phe Glu Asp Thr Leu  
485 490 495  
Asp Pro Thr

10

Claims

1. A composition of matter comprising an isolated nucleic acid sequence that encodes a human Cell Growth Regulatory Protein.

5

2. A composition of matter as described in claim 1 wherein said isolated nucleic acid sequence encodes a human Cell Growth Regulatory Protein with a transmembrane region.

10 3. A composition of matter as described in claim 2 wherein said isolated nucleic acid sequence encodes a human Cell Growth Regulatory Protein with a molecular weight of about 54.6kd.

4. A composition of matter comprising an isolated nucleic acid sequence as  
15 described in claim 3 wherein said nucleic sequence is a cDNA sequence.

5. A composition of matter as described in claim 4 wherein said isolated nucleic acid cDNA sequence encoding said human Cell Growth Regulatory Protein comprises an isolated nucleic acid fragment.

20

6. A nucleic acid sequence hybridizable under high stringency conditions to said nucleic acid sequence that encodes a human Cell Growth Regulatory Protein of claim 4.

25 7. A nucleic acid sequence that encodes a human Cell Growth Regulatory Protein, wherein said nucleic acid sequence is about 90% homologous to said nucleic acid sequence of claim 4.

8. A nucleic acid sequence that encodes a human Cell Growth Regulatory Protein,  
30 wherein said nucleic acid sequence is about 90% homologous to said nucleic acid sequence of claim 4.



9. Isolated host cells comprising an isolated nucleic acid sequence of claim 4 which encode a human Cell Growth Regulatory Protein.
10. Vectors that comprise an isolated nucleic acid sequence of claim 4 which encodes  
5 a human Cell Growth Regulatory Protein.
11. A nucleic acid sequence on deposit with the ATCC with accession no. 98169 which encodes a human Cell Growth Regulatory Protein.
- 10 12. A nucleic acid sequence that encodes a human Cell Growth Regulatory Protein, wherein said nucleic acid sequence is about 95% homologous to said nucleic acid sequence of claim 11 on deposit with the ATCC with accession no. 98169.
13. A nucleic acid sequence that encodes a human Cell Growth Regulatory Protein,  
15 wherein said nucleic acid sequence is about 90% homologous to said nucleic acid sequence of claim 11 on deposit with the ATCC with accession no. 98169.
14. A nucleic acid sequence that encodes a human Cell Growth Regulatory Protein, wherein said nucleic acid sequence encodes human Cell Growth Regulatory  
20 Proteins is selected from the groups consisting of Cell Growth Regulatory Protein-1b (residues 9-499), Cell Growth Regulatory Protein-1A (residues 58-499), Cell Growth Regulatory Protein-4b (residues 9-292), and Cell Growth Regulatory Protein- $\Delta$ T.M. (residues 9-499 with residues 379-398 deleted).
- 25 15. A composition of matter comprising a human Cell Growth Regulatory Protein.
16. A composition of matter as described in claim 15 wherein said human Cell Growth Regulatory Protein has a molecular weight of about 54.6 k daltons.
- 30 17. A composition of matter as described in claim 15 wherein said human Cell Growth Regulatory Protein is selected from the groups consisting of Cell Growth Regulatory Protein-1b (residues 9-499), Cell Growth Regulatory Protein-1A (residues

58-499), Cell Growth Regulatory Protein-4b (residues 9-292), and Cell Growth Regulatory Protein- $\Delta$ T.M. (residues 9-499 with residues 379-398 deleted).

18. Isolated host cells comprising an isolated nucleic acid sequence of claim 11  
5 which encodes a Cell Growth Regulatory Protein.

19. A complex comprising a substantially purified cell cycle target and said Cell Growth Regulatory Protein of claim 15.

10 20. A complex comprising a substantially purified cell cycle target and a Cell Growth Regulatory Protein selected from the groups consisting of Cell Growth Regulatory Protein-1b (residues 9-499), Cell Growth Regulatory Protein-1A (residues 58-499), Cell Growth Regulatory Protein-4b (residues 9-292), and Cell Growth Regulatory Protein- $\Delta$ T.M. (residues 9-499 with residues 379-398 deleted).  
15

21. A host cell comprising said complex of claim 19.

22. A host cell comprising said complex of claim 20.

20 23. A method of forming a complex comprising a cell cycle target and Cell Growth Regulatory Protein, comprising contacting in solution said cell cycle target with said Cell Growth Regulatory Protein, allowing sufficient time for said complex to form, and removing uncomplexed cell cycle target and Cell Growth Regulatory Protein.

25 24. Isolated antibody that binds to said Cell Growth Regulatory Protein of claim 15.

25. A pharmaceutical composition comprising a Cell Growth Regulatory Protein encoded by a nucleic acid on deposit with the ATCC, Accession Number 98169.

30 26. A process for producing a Cell Growth Regulatory Protein comprising culturing cells of claim 9 in a suitable culture medium and isolating said protein from said cells or said medium.

27. A method of identifying compounds that affect the interaction of a Cell Growth Regulatory Protein with a cell cycle target, comprising the steps of:

contacting said Cell Growth Regulatory Protein with said cell cycle target in the presence or absence of said compounds and under conditions that favor the phosphorylation of said cell cycle target; and

measuring the effect of said compounds on the phosphorylation of said cell cycle target.

28. A method as described in claim 27, wherein said Cell Cycle Growth Regulatory Protein is encoded by a nucleic acid sequence on deposit with the ATCC with accession no. 98169.

29. A method as described in claim 28, wherein said Cell Cycle Growth Regulatory Protein is encoded by a nucleic acid sequence that is about 95% or 90% homologous to said nucleic acid sequence on deposit with the ATCC with accession no. 98169.

30. A method of identifying compounds that affect the interaction of a Cell Growth Regulatory Protein with a cell cycle target having enzymatic activity, comprising the steps of:

contacting said Cell Growth Regulatory Protein with said cell cycle target in the presence or absence of said compounds and under conditions that favor the phosphorylation of said cell cycle target; and

measuring the effect of said compounds on the enzymatic activity of said cell cycle target.

31. A method as described in claim 29, wherein said Cell Cycle Growth Regulatory Protein is encoded by a nucleic acid sequence on deposit with the ATCC with accession no. 98169.

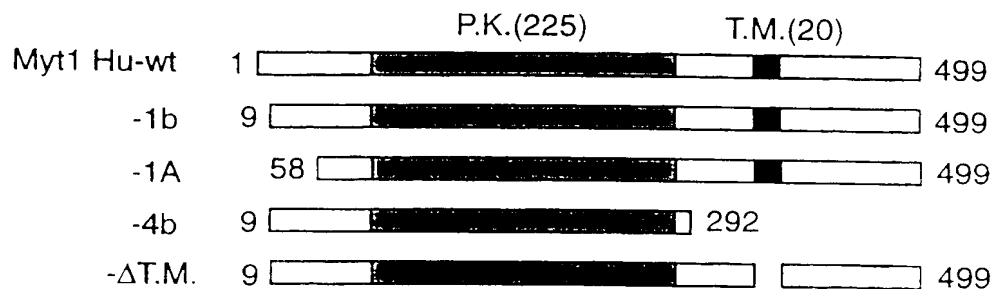
32. A method as described in claim 30, wherein said Cell Cycle Growth Regulatory Protein is encoded by a nucleic acid sequence that is about 95% or 90% homologous to said nucleic acid sequence on deposit with the ATCC with accession no. 98169.

1 / 2

FIGURE 1

## Human Myt1 Transmembrane Region

## pCMV-HA:MYT1 Constructs



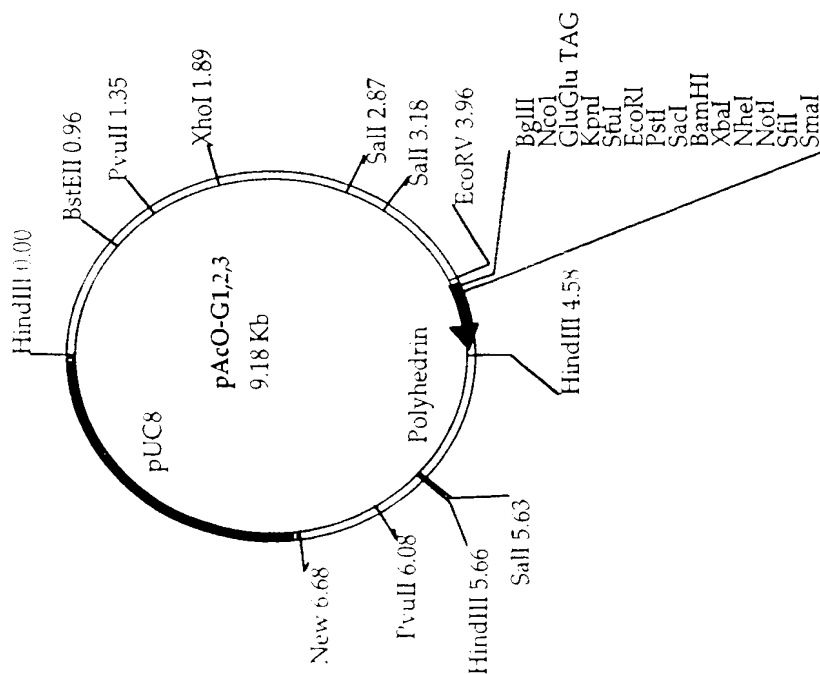


FIGURE 2

SEQUENCE OF MULTIPLE CLONING SITE

BglII NcoI GLUGLU TAG StuI EcoRI PstI SacI BamHI XbaI NheI NotI Sfi SmaI  
AAGATCTCCATGGCAATACATGCAATGGAAGGTAAGGCTGAAATTCCTGCAGAGCTCGAATCTCTAGAGCTAGCGGCCCGCCGGCCGACGACTCTGGCT - pAcO-G1

BglII NcoI GLUGLU TAG KpnI StuI EcoRI PstI SacI BamHI XbaI NheI NotI Sfi SmaI  
AAGATCTCCATGGCAATACATGCAATGGAAGGTAACAGGCCCTGAAATTCCTGCAGAGCTCGAATCTCTAGAGCTAGCGGCCCGCCGGCCGACGACTCTGGCT - pAcO-C2

BglII NcoI GLUGLU TAG KpnI StuI EcoRI PstI SacI BamHI XbaI NheI NotI Sfi SmaI  
AAGATCTCCATGGCAATACATGCAATGGAAGGTAAGGCTGAAATTCCTGCAGAGCTCGAATCTCTAGAGCTAGCGGCCCGCCGGCCGACGACTCTGGCT - pAcO-C3

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 97/18577

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/54 C12N15/86 C12N9/12 C12N5/10 C12Q1/48  
C07K16/40 A61K38/45

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12Q C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
----------	--	-----------------------

X	MILLS G.B. ET AL.: "Interleukin 2-induced tyrosine phosphorylation" J. BIOL. CHEM., vol. 265, no. 6, 25 February 1990, pages 3561-3567, XP002058139 see abstract	1-10, 15-17, 19-24, 26,27,30
---	--	---------------------------------------

X	ATHERTON-FESSLER S. ET AL.: "Cell cycle regulation of the p34/cdc2 inhibitory kinases" MOL. BIOL. CELL., vol. 5, no. 9, 1994, pages 989-1001, XP002057979 see abstract	15,16, 19,21, 23,24, 27,30
---	--	-------------------------------------

-/--

☒ Further documents are listed in the continuation of box C

☐ Patent family members are listed in annex

### Special categories of cited documents

- A. document defining the general state of the art which is not considered to be of particular relevance
- B. earlier document but published on or after the international filing date
- C. document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- D. document referring to an oral disclosure, use, exhibition or other means
- E. document published prior to the international filing date but later than the priority date claimed

- F. later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- G. document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- H. document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- I. document of the same patent family

Date of the actual completion of the international search

Date of issuing of the international search report

10 March 1998

14/03/1998

Name and mailing address of the ISA

European Patent Office - P.O. Box 1818 Patentlaan 2  
NL-1220 HV Rijswijk  
Tel: (+31-70) 340-2040, Tx: (+31-651) 6601  
Fax: (+31-70) 340-2016

Name of the ISA

ISA/EU

# INTERNATIONAL SEARCH REPORT

Int. Patent Application No.

PCT/US 97/18577

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	LIU F. ET AL.: "The human Myt1 kinase preferentially phosphorylates Cdc2 on Thr14 and localizes to the endoplasmic reticulum and Golgi complex" MOL. CELL. BIOL., vol. 17, no. 2, February 1997, pages 571-583, XP002057978 see the whole document	1-24, 26-32
A	-----	25
A	MUELLER P. ET AL.: "Myt1: a membrane-associated inhibitory kinase that phosphorylates Cdc2 on both Thr14 and Tyr15." SCIENCE, vol. 270, 6 October 1995, pages 86-90, XP002057980 cited in the application see abstract see figure 1 -----	1-32

